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TITLE: Tumor Suppression and Sensitization to Taxol-Induced Apoptosis of E1A in Breast Cancer Cells

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13. ABSTRACT (Maximum 200 Words)

The purpose of this project is to study the molecular mechanisms underlying E1A's proapoptotic effect and anti-tumor activity and to dissect the functional domains of E1A that are critical for its antitumor activity. Because a phase I E1A gene therapy protocol for human breast and ovarian cancers was completed and a phase II clinical trial is undergoing, we also plan to develop an alternative E1A mutant construct to maximize E1A therapeutic effects while minimizing its potential side-effects for cancer gene therapy.

In trying to understand the mechanism underlying E1A's antitumor activity, we have found that E1A downregulated VEGF expression both *in vitro* and *in vivo*, and mapped the domains required for this activity. We have also identified additional new target genes that were critically involved in E1A–mediated chemosensitization.

Also, we have been trying to identify other molecules that may be regulated by E1A via the genomic and proteomic approaches. These studies can provide useful information for us to better understand the molecular functions of E1A, and hopefully we can use this knowledge to better design a mutant E1A construct for cancer gene therapy in the future.

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Introduction:

The purpose of this project is to study the molecular mechanisms underlying E1A's proapoptotic effect and anti-tumor activity and to dissect the functional domains of E1A that are critical for its anti-tumor activity. Because a phase I E1A gene therapy protocol for human breast and ovarian cancers was completed and a phase II clinical trial is undergoing, we also plan to develop an alternative E1A mutant construct to maximize E1A therapeutic effects while minimizing its potential side-effects for cancer gene therapy in the future. The successful reconstruction of this mutant E1A gene will largely depend on our *in vitro* as well as *in vivo* studies of the different mutant stable cells.

Body

Statement of Work (no change):

Task 1: To test whether E1A could repress VEGF expression and whether or not mutation of the CR1 domain of E1A will abrogate this activity.

Task 2: To test whether CR1 domain of E1A represses VEGF transcription through recruitment of HDAC-1 via binding with p300 and pRB.

Task 3: To test whether E1A promotes apoptosis through its CR2 domain by disruption pRB-E2F-1 complexes, releasing free E2F-1.

Task 4: To test whether the E1A N-terminal deletion mutant, wither with or without combination of Taxol, can exhibit effects that are more beneficial than those of wild-type E1A in an orthodox breast cancer model in nude mouse.

A. Studies and Results:

In the past year we have continued to work on understanding the molecular mechanisms underlying E1A's proapoptotic effect and anti-tumor activity. Also we have been trying to identify other molecules that may be regulated by E1A via the genomic and proteomic approaches. This work may provide useful information for identification of novel target genes of E1A and new biomarkers as therapeutic targets. The progress for each aim will be discussed separately as following:

Task 1:

We have observed that stable expression of E1A do repress VEGF expression by Northern blot both in cell culture *in vitro* and in tumor tissue *in vivo* (Figure 1A and B). We also mapped the domains of E1A, which is required for repression of VEGF expression by analyzing of tissue samples obtained from tumors xenografts established by inoculation of vector control cells, wild-type E1A and different domain deletion mutant E1A stable cells (Figure 1C). We found that deletion mutation of an N-terminal fragment (ΔNT) of E1A enhanced the repression effect of E1A on VEGF expression in animal model *in vivo*.

Task 2:

We have been working on whether repression of VEGF by expression of E1A is achieved by inhibition of the histone accetyltransferase (HAT) activity of p300 via HDAC1. We have been working on whether expression of E1A could repress the HAT activity of p300. As was originally proposed in the proposal, we have obtained the critical materials for this Task, such as flag-tagged HDAC1 and Gal4-tagged Rb, from Dr. Douglas C. Dean, Washington University School of Medicine, St. Louis, Missouri. To test whether CR1 domain of E1A represses VEGF transcription through recruitment of HDAC-1 via binding with p300 and pRB, we used both antibody array and immunoprecipitation approaches to address this question (Figure 2). In antibody array study, we screened about 400 protein molecules and found that a few novel proteins were associated with E1A, which have not been reported yet. In addition, we also confirmed that some of the proteins that were reported to be associated with E1A in the literature were also positive by antibody array approach (Figure 2A). To confirm the above finding, we also selected a few molecules whose functions were known to be associated with tumor formation and/or apoptosis and used co-immunoprecipitation (IP) and Western blot analysis to test if they were bind with E1A. As shown in Figure 2B, the four proteins we selected do bind with E1A by co-IP analysis. To address whether

deletion mutation of E1A functional domains were affect its association with other proteins, we also did the antibody array in N-terminal deletion, CR1 deletion, and CR2 deletion mutant stable cells and function that mutation of these functional domains do affect E1A's association with a number of molecules (Figure 2C). Further analysis of these antibody array results, we found that N-terminal deletion mutant binds with HDAC1 more that wild-type E1A or any other deletion mutation mutants (Figure 3A), while deletion mutation of either N-terminal or CR1 domain did not affect E1A binding with pRB or pRB family member, pRB2 (p130). Deletion mutation of CR2 domain resulted in loss of binding with pRB and pRB2 (p130), however, this mutant turns out to bind with another pRB family member pRB1 (p110). Whether this is related to its anti-tumor effect in vivo needs further investigation. Since wild-type E1A and ΔCR2 mutant binds less HDAC1, suggest that the binding of E1A with HDAC1 is indirect and potentially through the binding with pRB. To test whether the altered binding with HDAC1 in ΔNT mutant will contribute to its enhanced activity in repressing the expression of VEGF through p300, we transiently transfected wild-type E1A and different domain deletion mutant stable cells with a Flag-tagged HADC1 construct and did a co-IP study by IP with anti-Flag antibody (M2) and Western blot with p300. We found that more p300 were recruited to HDAC1 in ΔNT cells than wild-type E1A or ΔCR1 and ΔCR2 mutant (Figure 3B), which is consistent with our study on VEGF expression in Figure 1C. These results suggest that repression of VEGF expression by E1A might be achieved by inhibition of the histone accetyltransferase (HAT) activity of p300 through its binding with pRB and subsequent recruitment of HDAC1.

Task3:

In the past year, we have been intensively working on the molecular mechanisms underlying E1A's proapoptotic effect. We have not yet been able to test the role of free E2F-1in E1A–mediated chemosensitization, because some of the antibodies we tried did not work well as we expected. However, in addition to the original proposed experiment we did identify additional two new target genes that were critically involved in E1A–mediated chemosensitization— we found E1A downregulated a critical oncogenic survival factor Akt while upregulated a proapoptotic factor p38. We demonstrated that activation of p38 and inactivation of Akt were necessary and sufficient for E1A–mediated sensitization to apoptosis induced by serum-starvation, ultraviolet (UV) -irradiation, tumor necrosis factor (TNF) -α, and different categories of anti-cancer drugs, such as adriamycin/doxorubicin, cisplatin, methotrexate, gemcitabine and paclitaxel (Taxol). By screening

human cancer cell lines and different types of tumor tissue samples and surrounding normal tissues, we found that the p38 pathway was deregulated in cancer cells due to elevated Akt activation. We also showed that block Akt activation results in elevated p38 phosphorylation and vice versa. We proposed and tested that a novel feed forward mechanism involving a protein phosphatase PP2A and ASK1, Akt, and p38 kinases regulated Akt and p38 activity, which can be turned on by E1A through upregulation of PP2A activity. A manuscript summarizing the above results was accepted for publication in **Molecular and Cellular Biology (MCB)**, which is attached for further description and for figures of this work. Also, a patent regarding this novel finding was filed (see appendices).

Recent reports published in Nature Genetics suggested a tumor suppressor role for p38, as inactivation of p38 by a protein phosphatase PPM1D (also called Wip-1) is involved in the development of human cancers by suppressing p53 activation (1,2). We also wanted to test if E1A could affect PPM1D activity. By screening several pairs of E1A stable cell lines established in human breast cancer, we found that expression of E1A do repress PPM1D protein expression compared with the respective vector transfected control cells (Figure 4A). In addition, we also found that repression of PPM1D expression was correlated with E1A-mediated activation of p38 activity and inactivation of Akt (Figure 4A). As PPM1D is also located within a breast cancer amplification epicenter (3), we are currently undergoing to determine if Akt will directly affect PPM1D phosphatase activity and indirectly affect p38 activation.

Task 4:

We are still working on the comparison of therapeutic efficacy of an N-terminal deletion mutant E1A with the wild–type E1A gene. The preliminary results we obtained from the animal model study showed that at least the N-terminal mutant of E1A worked as good as that of the wild-type E1A in terms of repression tumor growth and sensitization to chemotherapy *in vivo* in the context of a systemic gene therapy (Figure 5). Although we are not at the time to compare their effect on animal survival rate, we do observe a positive results in animals receiving a systemic gene therapy with N-terminal E1A mutant. We are currently repeating this experiment by including more animals in each treatment group and continue to monitor the difference of animal survival time in each group.

To gain a global view of genes and proteins regulated by E1A, we also used cDNA microarray and proteomic technologies including 2-D gel electrophoresis and Ciphergen protein chip array to identify

the target genes and proteins associated with E1A in breast cancer cells. Differentially expressed genes were found in E1A stable cells versus parental cells by either the cDNA microarray or 2-dimensional gel electrophoresis and Ciphergen protein chip technology (Figure 6). Althoguh these experiments are not directly related to the original proposal, they are relevant to E1A's proapoptotic effect and anti-tumor activity and to breast cancer. Therefore, we will continue to pursue on finding E1A associated molecules and dissect their function on tumor growth and apoptosis in breast cancer.

Key research accomplishments:

(1). We mapped domains of E1A responsible for downregulation of VEGF expression.

(2). Evaluated the chemosensitization effect of E1A by systemic E1A gene therapy approach. (See detail in attached Manuscript).

(3). We have found that upregulation of p38 activity and downregulation of Akt activity are necessary and sufficient for E1A–mediated sentization to apoptosis induced by serum–starvation, TNF-α, UV-irradiation, and different categories of anticancer drugs (MCB, in press, 2003).

(4). We have found that E1A downregulated a protein phosphatase PPM1D expression, which is amplified/overexpressed in human breast cancer.

(5). We identified that a few novel proteins associated with E1A by antibody array approaches and some of them have been also confirmed by co-IP and western blot analysis.

(6). We have found that deregulation of p38 activity through elevated Akt activation was a general phenomenon in human cancer, including breast and ovarian cancers (MCB, in press, 2003).

(7). We provided data to support that repression of VEGF expression by E1A might be achieved by inhibition of the HAT activity of p300 through binding with pRB and HDAC1 by antibody array and Co-IP approaches.

Reportable outcomes:

1. Manuscripts:

   Liao Y, Hung MC: "Regulation of p38 activity by Akt and its association with adenoviral E1A-mediated sensitization to apoptosis. " MCB, in press, 2003.

   Liao Y*, Zou YY, Xia WY, Hung MC: "Enhanced paclitaxel cytotoxicity and prolonged animal survival rate by non-viral mediated systemic delivery of E1A gene in orthotopic xonograft human breast cancer." Manuscript (to be submitted)

2. Abstracts and presentations:

Liao Y*, Zou YY, Xia WY, Hung MC: "Enhanced paclitaxel cytotoxicity and prolonged animal survival rate by non-viral mediated systemic delivery of E1A gene in orthotopic xonograft human breast cancer."

3. Patent (pending):

Mien-Chie Hung and **Yong Liao**: Compositions and methods for inactivating the Akt oncogene and activating the p38 pro-apoptotic gene. (MDA00-050)
Applied for coverage under U.S. Patent (Serial No: 60/277,788 and Application No. 10/103.542), Chinese Patent (No. 011117869), and Taiwanese Counterpart Patent (N0. 90106723)

Conclusion:

These studies on the molecular mechanisms underlying E1A's tumor suppression and chemosensitization can help us to better design an alternative E1A constructs for future gene therapy. Specifically, identification of additional E1A target genes, such as PPM1D, PP2A, Akt and p38, may help us in finding novel ways to treat cancer patients by targeting the deregulated signals. The combination of E1A gene therapy with Taxol or other chemotherapeutic drugs is one potential new therapeutic approach for the treatment of cancer patients, as we have shown in the animal models. The mutant E1A construct(s), if it works in animal model, could potentially be translated into the clinic and be of great benefit to breast cancer patients.

Reference:

1. Bulavin DV, Demidov ON, Saito S, Kauraniemi P, Phillips C, Amundson SA, Ambrosino C, Sauter G, Nebreda AR, Anderson CW, Kallioniemi A, Fornace AJ, Appella E. Amplification of PPM1D in human tumors abrogates p53 tumor-suppressor activity. Nat Genet. 2002 Jun;31(2):210-5.

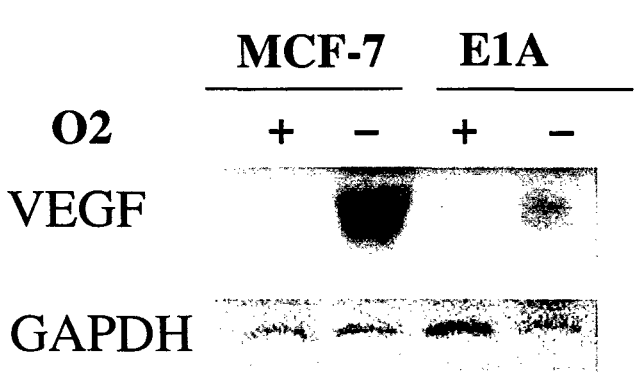
2. Li J, Yang Y, Peng Y, Austin RJ, Van Eyndhoven WG, Nguyen KC, Gabriele T, McCurrach ME, Marks JR, Hoey T, Lowe SW, Powers S. Oncogenic properties of PPM1D located within a breast cancer amplification epicenter at 17q23. Nat Genet. 2002 Jun;31(2):133-4.

3. Choi J, Appella E, Donehower LA. The structure and expression of the murine wildtype p53-induced phosphatase 1 (Wip1) gene. Genomics. 2000 Mar 15;64(3):298-306.

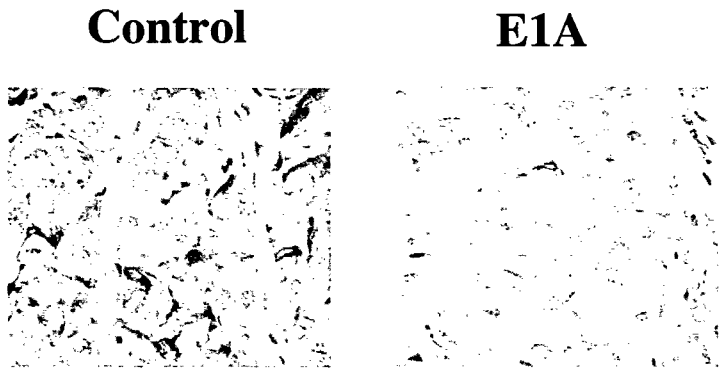
Appendices:

Figure 1. E1A downregulate VEGF expression *in vitro* (A) and *in vivo (B-C).*

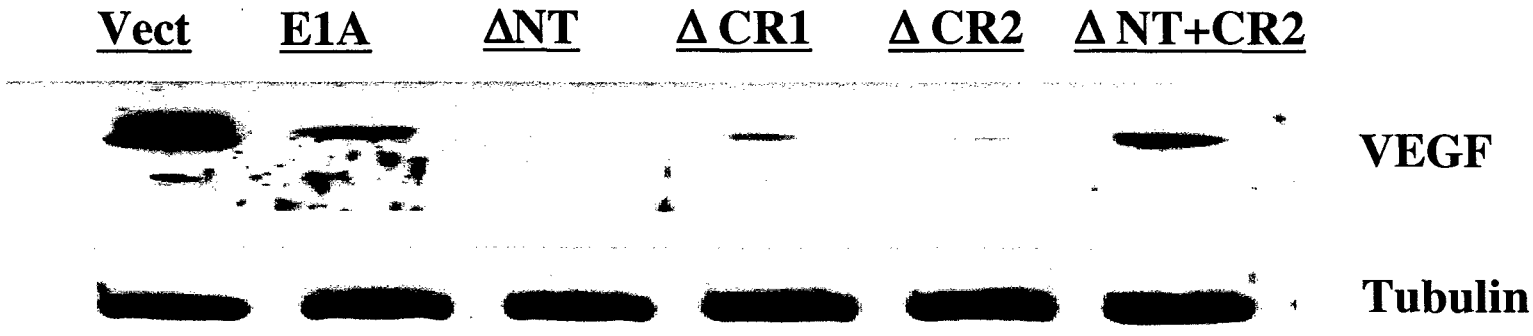
A. Northern Blot analysis of VEGF expression in cell culture *in vitro.*



B. Immunohistochemistry analysis of VEGF expression in tumor tissue *in vivo.*



C. Western blot analysis of VEGF expression in tumor tissues *in vivo* obtained from inoculation of vector control cells or wild-type E1A and different domain mutant E1A stable cells.



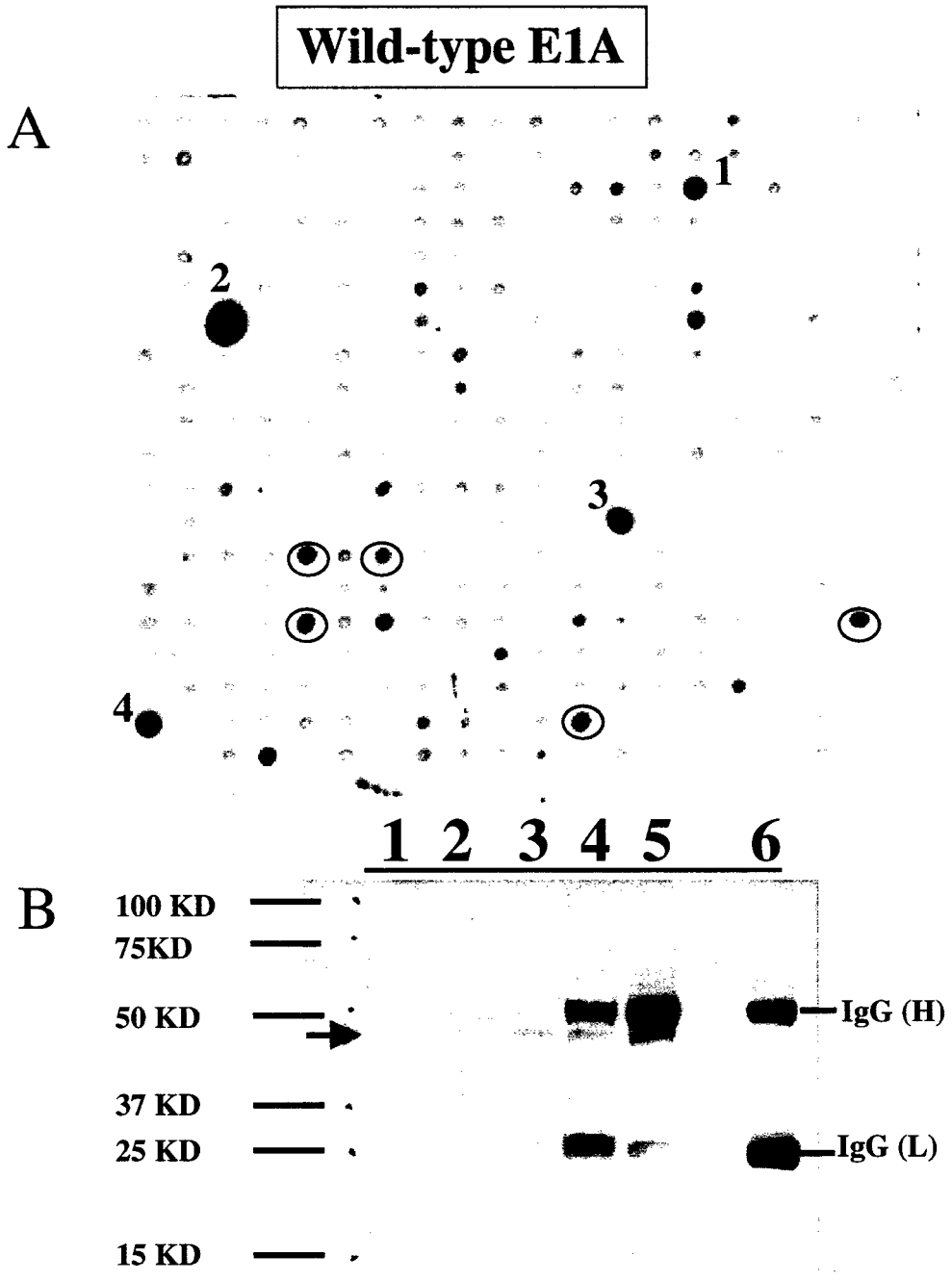
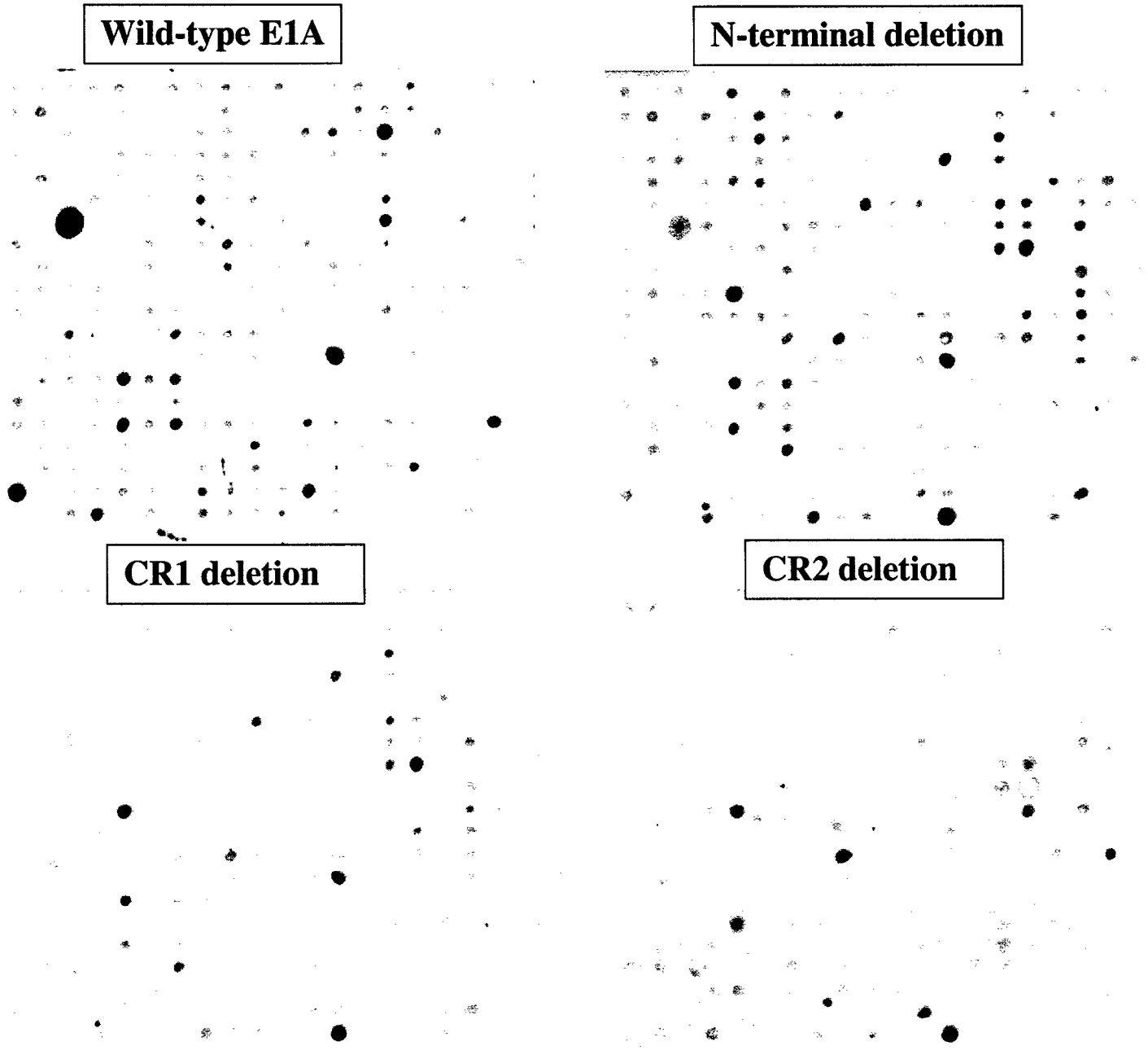


Figure 2

A. Antibody array of proteins associated with E1A. Number 1, 2, 3, and 4 represent proteins that have not been reported to be associated with E1A, but were identify by antibody array and their association with E1A were further supported by co-immuoprecipitation as shown in Figure B. Circled proteins are those previously reported to be associated with E1A.

B. Proteins marked as 1 to 4 in Figure A were immunoprecipitated by respective antibodies and bloted against anti-E1A antibody (M73). Number 5 represents a positive control by using anti-E1A antibody for the immunoprecipitation. Number 6 represents a negative control by using normal IgG. Arrow indicates the E1A protein band.

C


Wild-type E1A
N-terminal deletion
CR1 deletion
CR2 deletion

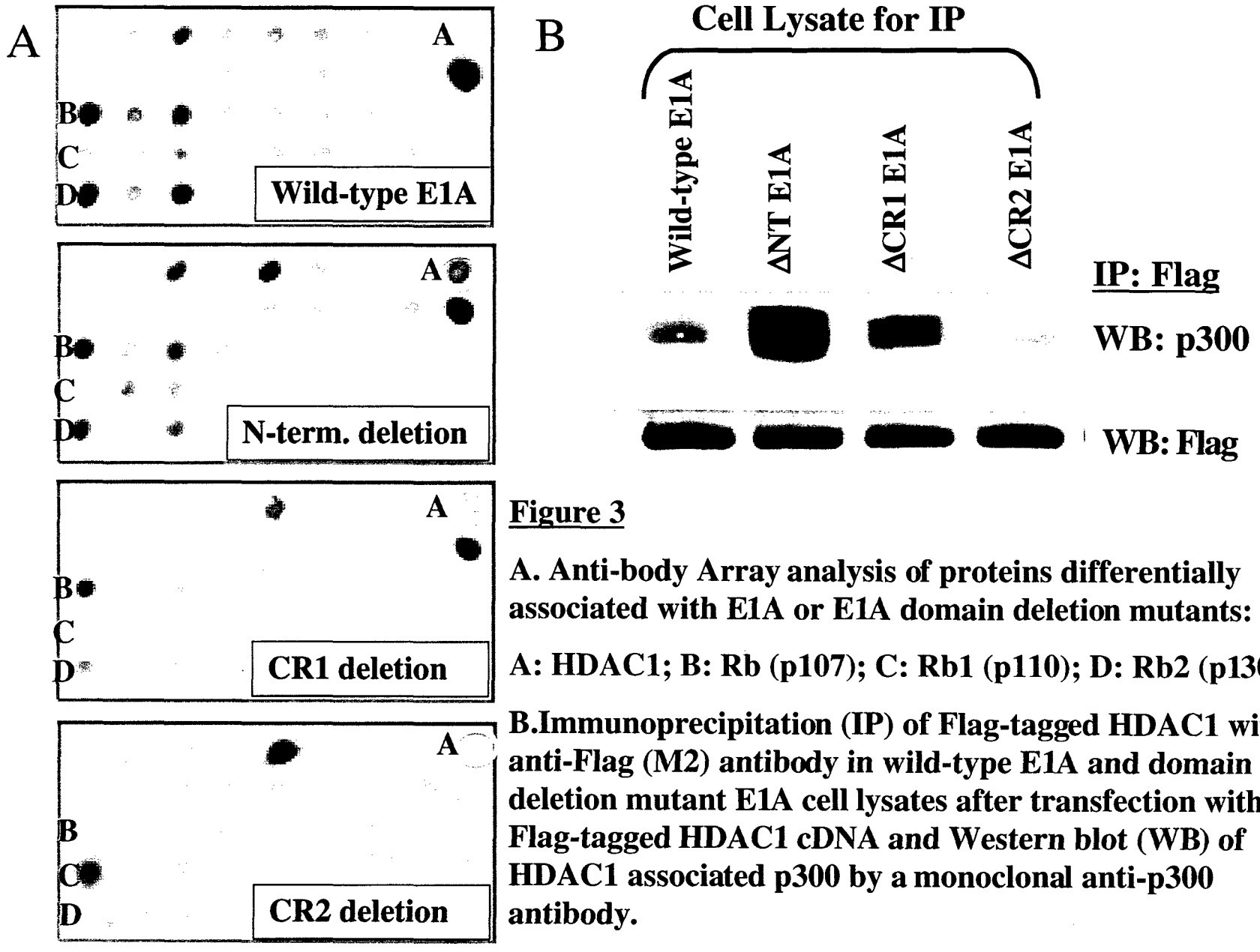


Figure 3

A. Anti-body Array analysis of proteins differentially associated with E1A or E1A domain deletion mutants:

A: HDAC1; B: Rb (p107); C: Rb1 (p110); D: Rb2 (p130)

B.Immunoprecipitation (IP) of Flag-tagged HDAC1 with anti-Flag (M2) antibody in wild-type E1A and domain deletion mutant E1A cell lysates after transfection with Flag-tagged HDAC1 cDNA and Western blot (WB) of HDAC1 associated p300 by a monoclonal anti-p300 antibody.

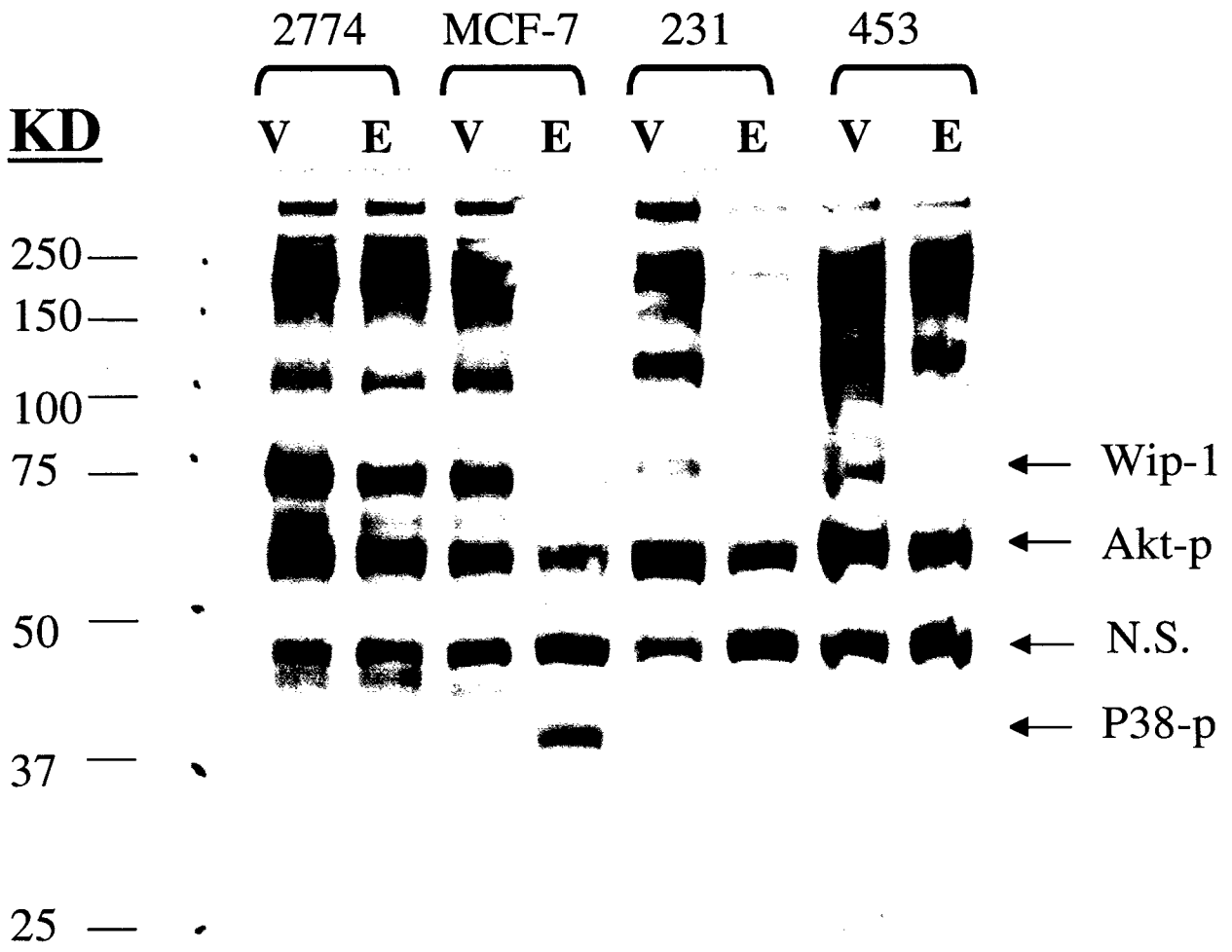


Figure 4 **Repression a protein phosphatase PPM1D (Wip-1) expression by E1A in stable E1A expression cells correlates with its effect on Akt and p38 kinase phosphorylation in breast cancer 2774, MCF-7, MDA-MB-231, and MDA-MB-453.**

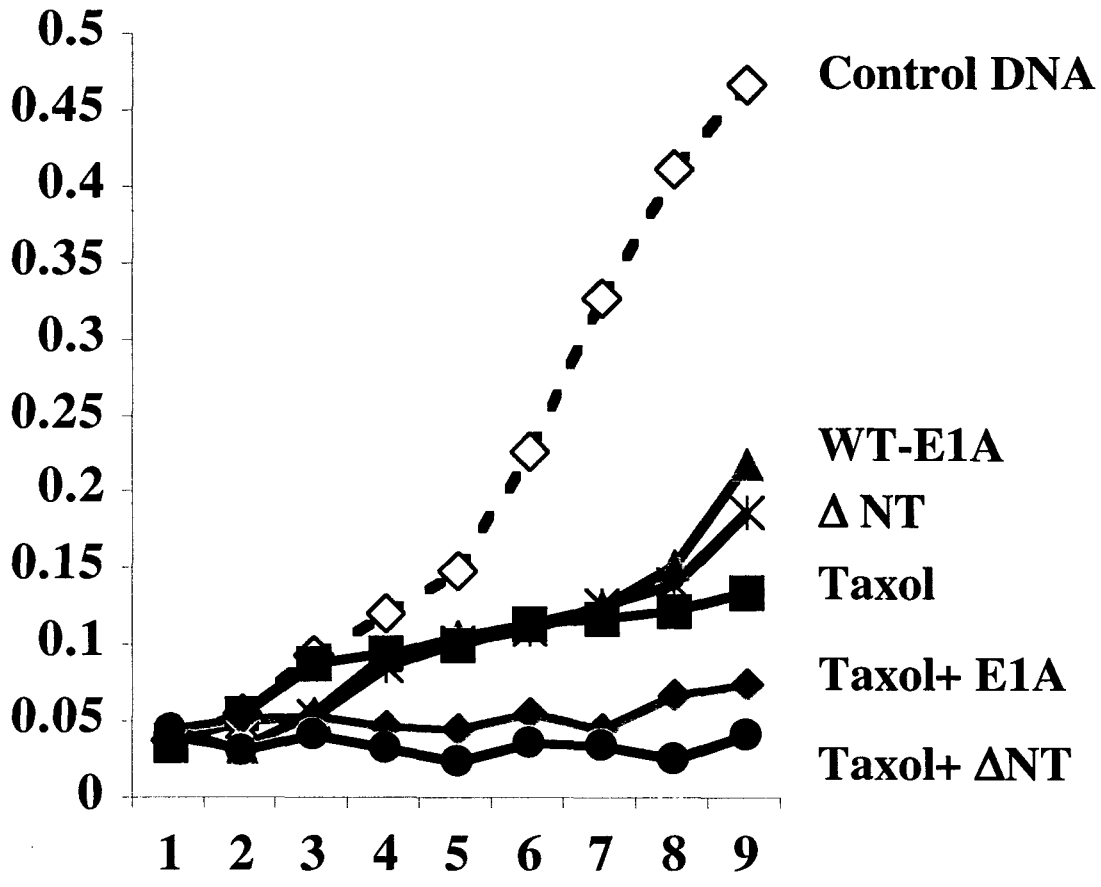
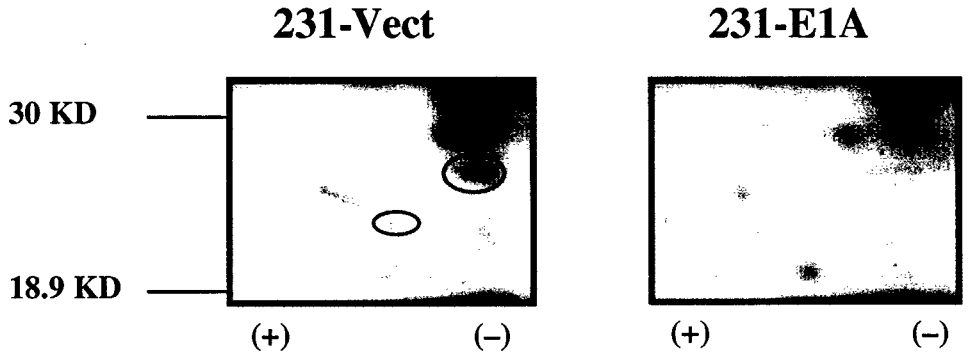


Figure 5. Systemic gene therapy effect of wild-type E1A or N-terminal deletion mutant E1A with or without combination with paclitaxel (Taxol) chemotherapy in breast cancer xenograft established by inoculation with MDA-MB-231 cells in nude mice.

Figure 6

Proteins differentially expressed in 231-E1A stable cells identified by using 2D gel (A) and Ciphergen protein chip profiling analysis (B)

A. E1A down-regulated proteins identified by 2D gel electrophoresis.



B. Differentially expressed proteins in 231-E1A cells identified by Ciphergen proteinchip analysis

Type of Protein chip	Up-regulated proteins (KD)	Down-regulated proteins (KD)
H4	37.5, 43.7, 48.8, 53.1, 87.4, 4.36, 6.81	45.4, 56.5, 65.7, 69.7, 6.25
SAX2	34.9, 61.1, 7.9	12.4, 17.5, 25.9, 148.8, 6.18
WCX2	37.4, 3.3	69.7
IMAC-cu	37.3, 40.6	49.6, 65.7

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MCB 367-03 Revised

Dear Dr. Hung:

I am pleased to inform you that your manuscript has been accepted for publication in *Molecular and Cellular Biology* and is being forwarded to ASM today.

Sincerely,

Jean Y.J. Wang, Ph.D.
Editor

MCB 367-03 Revised

Regulation of the activity of p38 mitogen-activated protein kinase by Akt in cancer and adenoviral protein E1A-mediated sensitization to apoptosis

Running title: Activation of p38 by adenoviral E1A through repression of Akt

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Abstract:	187 words
Materials and Methods:	1,190 words
Combined:	4,374 words
Introduction:	532 words
Results:	2,641 words
Discussion:	1,201 words

The adenoviral early region 1A (E1A) protein mediates sensitization to different stimuli–induced apoptosis, such as tumor necrosis factor (TNF)–α, UV– and γ–irradiation, and different categories of anti-cancer drugs. However, the molecular mechanisms underlying E1A–mediated sensitization to apoptosis are still not completely defined. Here, we show that E1A mediated sensitization to apoptosis by the inactivation of a key survival factor Akt and the activation of a pro-apoptotic factor p38. Also inactivation of Akt by either a specific inhibitor or a genetic knock out of Akt1 results in p38 activation, possibly, through the release of the activity of p38 upstream kinases, including ASK1 and MEKK3. In addition, we showed that p38 phosphorylation is downregulated and Akt phosphorylation is upregulated in multiple human tumor tissues and this correlates with tumor stage in human breast cancer. A deletion mutation of a conserved domain of E1A, which is required for E1A–induced downregulation of Akt activity, disrupts E1A–mediated upregulation of p38 activity and also abrogates E1A–mediated chemosensitization. Thus, activation of p38 and inactivation of Akt may have general implications for tumor suppression and sensitization to apoptosis.

Many types of tumors are associated with activated oncogenic kinases and two complementary roles of these oncogenic kinases are to stimulate signaling pathways that enable cells to function independent of their environment and to make tumor cells resistant to genotoxic therapies, such as chemo- and radiotherapy (22, 24, 48). Deregulated growth signaling pathways and acquired resistance toward apoptosis therefore constitute two hallmarks of most, if not all, human tumors (18). For example, it has been shown that the serine/threonine kinase Akt and its family members Akt 2, and 3 are either amplified or their activity is constitutively elevated in human carcinomas such as breast, pancreatic, ovarian, brain, prostate, and gastric adenocarcinomas (39, 50). As it is a direct downstream target of phosphoinositol 3-kinase (PI3K), Akt is also a key oncogenic survival factor and can phosphorylate and inactivate a panel of critical proapoptotic molecules, including Bad, caspase-9, the Forkhead transcription factor FKHRL1 (known to induce expression of pro-apoptotic factors such as Fas ligand), GSK3-β, cell cycle inhibitors p21 and p27, and tumor suppressor TSC2, etc. (4, 25, 39, 50, 58). Akt can also inactivate p53, a key tumor suppressor, through phosphorylation and nuclear localization of MDM2 (33, 50, 59). Activation of Akt has been shown to induce resistance to apoptosis induced by a range of drugs (41). Thus, molecules that can block Akt activity may have important significance in cancer therapy and drug sensitization.

The adenovirus E1A induces chemosensitization among different categories of anti-cancer drugs, including cisplatin, adriamycin, etopside, staurosporine, 5-fluorouracil, and paclitaxel (Taxol) (5, 14, 16, 32, 45, 53), suggesting that a general cellular mechanism may exist to regulate E1A–mediated chemosensitization. However, the molecular mechanisms underlying E1A-mediated chemosensitization are still not completely

defined. Earlier studies on normal fibroblast cells revealed that E1A-mediated sensitization to cytotoxic anti-cancer drugs depends on the expression of functional p53 and p19ARF, an alternative splicing form of p16INK4a (12, 31, 32). E1A was also shown to downregulate Her-2/neu overexpression and facilitate E1A-mediated sensitization to the cytotoxicity of anticancer drugs in human breast and ovarian cancer cells (53, 55, 56). In another study, E1A was reported to mediate sensitization to anti-cancer drugs in human osteosarcoma cells (16) in a p53– and Her-2/neu–independent manner. Similarly, there is no correlation between p53 protein level and sensitivity of DNA-damaging agents in keratinocytes carrying adenovirus E1A (45). A few other critical molecules were also proposed to be involved in E1A-induced chemosensitization, such as the pro-apoptotic protein Bax, caspase-9, or a yet unidentified inhibitor that ordinarily provides protection against cell death (14, 15, 34, 43, 49, 52). However, none of the above molecules or pathways can really serve as a general cellular mechanism for E1A-mediated sensitization to apoptosis in a diverse cellular context. Recently, transcriptional upregulation of pro-caspases (such as pro-caspase-3, -7, -8, and –9) through E1A-mediated disruption of pRB function and subsequent release of free E2F-1 was reported to contribute to both p53-dependent and p53-independent drug sensitization by E1A in diploid normal fibroblast cells (37). In the current study, we found that E1A can activate p38 and inactivate Akt and showed that this pathway may provide a general cellular mechanism for E1A to mediate sensitization to different categories of anti-cancer drugs.

MATERIALS AND METHODS

Cell Culture, Cell Harvest, and Western blot. Human breast, ovarian, prostate, pancreatic, and colon cancer cell lines were grown in Dulbecco's modified Eagle's medium/F-12 (Life Technologies Inc., Rockville, MD) supplemented with 10% fetal bovine serum. The stable E1A-expressing cell lines in breast cancer MDA-MB-231 and MCF-7 were established as described previously (36, 52). Similarly, domain deletion mutant constructs of E1A were transfected into MDA-MB-231 cells and stable clones were screened and selected in the presence of G418. Akt1 knockout MEF cells and myristoylated, membrane-bound, constitutively active Akt1 (myr-Akt)–transfected stable Rat1 cells were provided by Dr. Nissim Hay (University of Illinois at Chicago, Chicago, Illinois) (10).

For the analysis of basal Akt and p38 expression and activity, cells were serum-starved overnight before harvesting. Cells were then washed twice with cold PBS and lysed in a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate (NaVO3), and 1.5% aprotinin. The cell extracts were clarified by centrifugation, and protein concentrations were determined using a Bio-Rad (Hercules, CA) protein assay reagent and analyzed in a spectrophotometer using bovine serum album (Sigma, St. Louis) as the protein standard. Aliquots of protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Millipore Corp., Bedford, MA) using standard procedures. The membranes were then subjected to western blotting, and the blots were

developed with the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ).

Primary antibodies. For western blot analysis, rabbit polyclonal antibodies against phospho-Akt (Ser 473, Cat. No. 9271; 1: 1000 dilution) and phospho-p38 (Thr 180/Tyr 182, Cat. No. 9211; 1: 1000 dilution) were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal antibodies against non-phosphorylated total Akt (Cat. No. 9272; 1: 500 dilution), p38 (Cat. No. 9212; 1: 500 dilution), and cleaved PARP (Asp214, Cat. No. 9541; 1: 1000 dilution) were also from Cell Signaling Technology. A rabbit polyclonal antibody against beta-actin was used as a loading control for western blot and was purchased from Sigma. For immunohistochemical (IHC) study, we used an IHC specific rabbit polyclonal antibody against phospho-Akt (Ser 473, Cat. No. 9277) or an IHC specific monoclonal antibody against phospho-p38 (Thr180/Tyr 182, Cat. No. 9216) at 1: 75 dilution for both antibodies. The monoclonal antibody used against the E1A proteins was M58 (PharMingen, San Diego, California). Rabbit polyclonal anti-Bax antibodies, a hamster antihuman Bcl-2 monoclonal antibody, and rabbit polyclonal anti-ASK1 (H-300) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, California). Mouse monoclonal antibodies against human caspase–3 and –7 were from Transduction Laboratories (1:1000; C31720, Lexington, KY) and BD PharMingen (1:1000; 66871A, San Diego, CA), respectively. Rabbit polyclonal antibodies against human caspase–8 and –9 were from Santa Cruz Biotechnology (1:500; SC-7890/H-134, Santa Cruz, CA) and Cell Signalling Technology (1:500; #9502, Beverly, MA), respectively. To detect HA-tagged proteins, a monoclonal anti-HA antibody was used (1:1000; Cat. No. 1 583 816; Boerhinger Mannheim,

Indianapolis, Indiana). A monoclonal anti-FLAG antibody (M2) was purchased from Sigma (1:1000; St. Louis, Washington).

Transient transfection, MTT assay, luciferase assay, and FACS analysis. The standard MTT assay was performed to measure the viable cells after treatment with anti-cancer drugs as described previously (52). Expression vectors for HA-p38, CA-Akt, DN-Akt, and cytomegalovirus driving luciferase (pcDNA3-Luc) were used in this study. First, 1 x 10^5 cells in a 60-mm–well dish were transfected with 2.2 μg of total DNA using the DC-Chol cationic liposome as described previously (52). After 48 hr, the cells were split into three sets: one used for a luciferase assay after exposure with or without Taxol for 24 hr, one used to analyze Akt and p38 protein expression, and one fixed in 75% ethanol, stained with propidium iodide (25 μg/ml), and sent for FACS analysis. The percentage of Taxol-treated cells that exhibited luciferase activity was normalized using the luciferase activity of the untreated cells as the baseline (100%). Standard deviations from three independent experiments were calculated.

Establishment of IPTG-inducible DN-p38 stable cell lines. One E1A-expressing MDA-MB-231 clone was cotransfected with an IPTG-inducible dominant negative (DN)-p38α construct (a gift from Philipp E. Schere, Albert Einstein College of Medicine, Bronx, New York) and the plasmid pCMVLacI (Stratagene, La Jolla, California). Stable clones were selected in the presence of 200 μg/ml hygromycin.

Immunoprecipitation. After transient transfection with HA-tagged p38 or CA-Akt, cells were stimulated using 10 μM insulin for 15 min. Cells were then lysed, and cell lysates were centrifuged at 14,000 rpm for 30 min. The supernatants were then transferred to a fresh tube. Proteins were cleared via addition of a normal mouse or rabbit

IgG and immunoprecipitated with anti-p38, anti-Akt, or anti-HA antibodies. Immunoprecipitates were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. Akt, p38, and ASK1 were detected using Western blotting.

Kinase assay. Nonradioactive kinase assay kits for p38 and Akt were purchased from Cell Signaling (New England BioLabs, Beverly, Massachusetts). The p38 and Akt kinase activities were measured according to the manufacturer's protocol using GST-ATF-2 as the substrate for p38 and GST-GSK-3-β as the substrate for Akt.

Tissue microarray and Immunohistochemistry. Tissue microarray slides (HistoArray #IMH-343/BA2 and IMH-304/CB2) were purchased from IMGENEX (San Diego, CA). Detailed information about each slide is available on-line (http://www.imgenex.com/histoarrays/). Slide procession and immunohistochemistry staining were performed according to the manufacturer's protocol. Briefly, Tissue slides were heated at 60 ^{0}C, deparaffinized in xylene, hydrated in graded ethanol and then immersed in tap water. Antigen retrieval was performed with 0.01M citrate buffer at pH 6.0 for 20 min in a 95 ^{0}C water bath. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide solution followed by three sequential PBS washes (5 min each). Slides were then blocked by respectively normal serum of each primary antibody and followed by incubation with primary antibody diluted in TBS-T (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 1% ovalbumin and 1 mg/ml sodium azide followed by incubation with biotinylated second antibody for 30 min at room temperature, washed with PBS again and followed by incubation with Avidin-Biotin Complex (Vestastain Elite ABC kit from Vector Laboratories, Inc., Burlingame, CA, USA). Slides were washed with PBS again, and incubated with AEC (3-amino-9-

ethylcarbazole) substrate kit (Cat. No. Sk-4200, Vector Laboratories, Inc.), counterstained in Meyer's xylene.

One representative slide per case was evaluated with the antibodies mentioned above. The intensities of staining seen in different areas of the same slide were analyzed according to criteria described previously in the literature (1). The intensity is designated as 0 when no cells stain, 1+ when 10-20% of cells stain (weak), 2+ when 20-50% of cells stain (moderate), and 3+ when more than 50% of cells stain (strong).

Statistics. For statistical analysis, groups scored as 0 and 1+ were combined as weak staining, while groups scored as 2+ and 3+ were combined as strong staining. Similarly, to simplify the statistical analysis, breast tumors with stage 1 and 2 were combined as early stages of tumors (n=25 cases), while tumors with stage 3 and 4 were combined as late stages of tumors (n=25 cases). Statistical analysis was performed by using $\chi 2$ analysis.

RESULTS

E1A upregulates p38 activity and downregulates Akt activity. To determine whether apoptosis-related kinases are involved in E1A-mediated sensitization to apoptosis, we examined the phosphorylation status of three well-known kinases involved in regulation of apoptosis – p38, Akt, and JNK – in E1A-expressing MDA-MB-231 and MCF-7 cells (231-E1A and MCF-7-E1A) versus vector-transfected cells (231-Vect, MCF-7-Vect). We detected phosphorylated p38 in cells stably expressing E1A but not in vector-transfected cells. However, the level of phosphorylated Akt was much higher in vector-transfected cells than in E1A-expressing cells. The levels of total Akt and p38

were similar in both types of cells (Fig. 1A). Kinase assays showed that p38 activity was higher and Akt activity was lower in 231-E1A and MCF-7-E1A cells than in 231-Vect and MCF-7-Vect cells (Fig. 1B and C). We did not detect any difference in the level of phosphorylated JNK between E1A-expressing and vector-transfected cells (data not shown). These results indicated that E1A enhanced the activity of the proapoptotic kinase p38 and repressed the activity of the anti-apoptotic kinase Akt, but did not affect JNK phosphorylation.

A recent report showed that transient transfection of E1A resulted in the accumulation of caspase proenzymes in human normal diploid fibroblasts (37). We therefore compared the expression levels of pro-caspase proenzymes caspase-3, -7, -8, and –9 between E1A transfectants and vector transfected carcinoma cells, including breast cancer MDA-MB-231, MDA-MB-453, MCF-7 and ovarian cancer 2774 cells. Unlike what was demonstrated in normal fibroblast cells, we did not observe a unanimous increase of these caspase proenzymes in the E1A stable cells established in human cancer cells with epithelial origin (Fig. 1D). This suggests that transcriptional upregulation of the caspase proenzymes in these human cancer cells may not be as critical as it is in the normal fibroblast cells and other cellular mechanisms may exist for the E1A–mediated sensitization to apoptosis in human cancer cells.

Upregulation of p38 activity and downregulation of Akt activity correlate with E1A–mediated sensitization to Taxol–induced apoptosis. To test whether alteration of the kinase activity of Akt or p38 played a role in E1A-mediated sensitization to apoptosis, we compared the kinetics of phosphorylation of Akt or p38 with paclitaxel (Taxol)–induced apoptosis in 231-E1A cells, using PARP cleavage and Bcl-2

phosphorylation as apoptotic cell death markers. PARP cleavage and Bcl-2 phosphorylation occurred after decreased Akt phosphorylation and increased p38 phosphorylation in 231-E1A cells after exposure to 0.01 μM Taxol (Fig. 2A). However, no significant change was detected in the protein levels of p53 and Bax (Fig. 2A). The same concentration of Taxol did not trigger PARP cleavage, induce Bcl-2 phosphorylation, or modulate the levels of phosphorylated p38 and Akt in the parental MDA-MB-231 cells (data not shown). To trigger a similar response in parental MDA-MB-231 cells, a much higher dosage was required (Fig. 2B). The results suggest that downregulation of Akt and upregulation of p38 activities may be involved in the E1A-mediated sensitization to Taxol-induced apoptosis.

Activating p38 and inactivating Akt are required for E1A-mediated sensitization to drug-induced apoptosis. To evaluate whether activation of p38 is required for E1A-mediated sensitization to Taxol, we tested whether blocking p38 activity could inhibit E1A-mediated sensitization in 231-E1A cells. We used the specific p38 inhibitor SB203580 (Fig. 3A) and a dominant-negative p38 (DN-p38) mutant to block p38 activation (Fig. 3B). A pcDNA3-Luciferase (pcDNA-Luc) construct was transfected into 231-E1A cells and luciferase activity was used as a measurement for cell survival. Pretreatment with SB203580 inhibited the phosphorylation of p38 in cells with or without exposure to Taxol (Fig. 3C, lanes 3–4) and protected from a Taxol–induced decrease of luciferase activity (Fig. 3C, lane 2 vs. 4). In addition, FACS analysis showed that pretreatment using SB203580 protected 231-E1A cells from Taxol-induced apoptosis (27.5% vs. 18.0%) (Fig. 3D, lanes 2 and 4, bottom). These data suggest that p38 activation is required for E1A-mediated sensitization to Taxol-induced apoptosis. Using a dominant

negative p38 (DN-p38) to block p38 activation further supported the above results (Fig. 3B). When the cells were switched to medium containing 5 μM IPTG for 24 hr, expression of IPTG-inducible DN-p38 was induced in the presence or absence of Taxol (Fig. 3B, lower panel). FACS analysis showed that induction of DN-p38 by IPTG significantly inhibited Taxol-induced apoptosis (Fig. 3B, upper panel). Identical results were obtained when two additional stable clones were studied (data not shown). However, IPTG could not induce this effect in the 231-E1A cells without IPTG-induced DN-p38 (data not shown). Taken together, these data suggest that p38 activation is required for E1A-mediated sensitization to Taxol–induced apoptosis.

To determine whether downregulation of Akt activity is also required for E1A-mediated sensitization to Taxol, we examined whether activation of Akt by transfection of a constitutively active Akt construct (CA-Akt) would inhibit Taxol-induced apoptosis in 231-E1A cells. The level of phosphorylated Akt and luciferase activity was increased in CA-Akt–transfected 231-E1A cells as compared with that in the control 231-E1A cells (Fig. 3C, lanes 1 and 2 vs. 5 and 6). FACS analysis showed that fewer apoptotic cells were detected in CA-Akt–transfected cells (15.9%) than in control 231-E1A cells (27.5%) after exposure to Taxol (Fig. 3D, lane 2 vs. lane 6, bottom). Thus, inhibition of Akt phosphorylation is also required for E1A-mediated sensitization to Taxol-induced apoptosis.

Activating p38 and inactivating Akt represent a general cellular mechanism in response to different apoptotic stimuli. To determine whether the same mechanism of E1A-mediated sensitization to Taxol applies to other anticancer drugs, we tested the effects of four additional drugs used in the clinic for treatment of human cancer. These

four anti-cancer drugs induce anti-tumor activities through different modes of action: doxorubicin/adriamycin (topoisomerase II inhibitor), cisplatin (DNA-damaging agent), methotrexate (antimetabolite drug), and gemcitabine (antimetabolite drug). Expression of E1A significantly enhanced each drug's cytotoxicity in MDA-MB-231 cells, determined using the MTT assay (Fig. 4A). In addition, downregulation of Akt phosphorylation and upregulation of p38 phosphorylation and PARP cleavage were observed in 231-E1A cells but not in 231-Vect cells treated with each of the drugs at the same dosage (Fig. 4B). These results suggest that activation of p38 and inactivation of Akt may contribute to E1A-mediated sensitization to apoptosis induced by these different drugs.

To address whether activation of p38 and inactivation of Akt also applied to drug-induced apoptosis in the absence of E1A, we tested whether increasing the dosage of gemcitabine or adriamycin could also enhance p38 activation and inhibit Akt activation, which would then contribute to drug-induced apoptosis. When MDA-MB-231 and HBL-100 cells were exposed to a dose ten times higher of gemcitabine or adriamycin than used in the Fig. 4B, we observed a similar pattern of downregulation of Akt and upregulation of p38 activation, which was correlated with PARP cleavage (Fig. 4C). Similar results were also observed in MDA-MB-231 cells when exposed to a higher dose of Taxol (Fig. 2B). These results suggest that p38 activation and Akt inactivation may not be limited to E1A-mediated sensitization to apoptosis, but may also contribute to drug-induced apoptosis in the absence of E1A. Thus, downregulating Akt activity and upregulating p38 activity may represent a general cellular mechanism of response to apoptotic stimuli, and E1A may turn on this cellular mechanism and mediate sensitization to drug–apoptosis.

To determine the physiological relevance of inactivation of Akt and activation of p38 in the execution of apoptosis, we extended our investigation to apoptosis induced by serum starvation, TNF-α, and UV–irradiation. We observed that phosphorylation of p38 and dephosphorylation of Akt were correlated with serum starvation–, TNF-α–, and UV–induced PARP cleavage in 231-E1A cells, especially in detached apoptotic cells (Fig. 4D, lanes 1-8). However, a dose of 10 times higher is required for inducing a similar response in parental MDA-MB-231 cells as in 231-E1A cells (Fig. 4D, lanes 9~11). Taken together, downregulation of Akt activation and upregulation of p38 activation may also represent a general cellular mechanism in response to different apoptotic stimuli.

The physiological regulation of p38 activity by Akt is through ASK1 and MEKK3, the upstream kinases of p38. The above results suggest that both downregulation of Akt and upregulation p38 activities are involved in E1A-mediated sensitization to apoptosis. We noticed that reduced Akt phosphorylation occurs before enhanced p38 phosphorylation in the kinetic study of E1A-mediated sensitization to Taxol–induced PARP cleavage (Fig. 2A). We therefore asked whether Akt may act upstream of p38. To this end, Akt activity was blocked by either a specific PI3K inhibitor Wortmannin or a genetic method to knock out Akt expression. Blocking Akt activation using Wortmannin in MDA-MB-231 cells resulted in decreased Akt phosphorylation and increased p38 phosphorylation (Fig. 5A). And when Akt phosphorylation was recovered, the p38 phosphorylation was reduced again (please see 8-24 hrs time points). These results indicate that Akt phosphorylation was required for repressing p38 activation, suggesting that the former is upstream from the later. This conclusion was further supported by the study on Akt1 knockout mouse embryo fibroblast (MEF) cells and myr-

Akt1 transfected stable cells. We observed that the level of phosphorylated p38 was increased in Akt1 (–/–) MEF cells compared with that in Akt (+/+) and Akt (+/–) MEF cells. Furthermore, the phospho-p38 protein was undetectable in the Akt constitutively activated myr-Akt1 stable cells (Fig. 5B). These results indicate that Akt is able to inhibit p38 activity.

In an attempt to determine how Akt regulated p38, we sought to determine whether Akt is physically associated with p38 using coimmunoprecipitation experiments. We did not detect p38 in immunoprecipitated Akt samples (Fig. 5C) or Akt in immunoprecipitated p38 samples (data not shown), suggesting that Akt and p38 were not directly associated under the conditions we used. A recent report demonstrated that ASK1 is a substrate of Akt (28) and ASK1 has been shown to be an upstream kinase of p38 (23, 51), suggesting that Akt may indirectly regulate p38 activity through ASK1. Indeed, we also detected that ASK1 was coimmunoprecipitated with Akt in our experimental system (Fig. 5C). To test if Akt may downregulate p38 activation through the repression of p38 upstream kinases, such as ASK1, we blocked the activity of either ASK1 or MEKK3, both of which are p38 upstream kinases that can be inactivated by Akt (17, 28), by using a kinase-dead, dominant negative mutant of ASK1(DN-ASK1) or MEKK3 (DN-MEKK3). As expected, blockade of either ASK1 or MEKK3 activity by DN-ASK1 or DN-MEKK3 repressed p38 phosphorylation and its kinase activity, as measured by phosphorylation of GST-ATF-2 fusion protein in a dose-dependent manner in Akt1 (–/–) MEF cells (Fig. 5D), suggesting that Akt inhibits p38 activation through repression of ASK1 and/or MEKK3 activation. Unlike what we expected, however, the combination of DN-ASK1 and DN-MEKK3 did not results in an additional reducion of p38 kinase

activity or phosphorylation of p38 in these MEF Akt (-/-) cells. These data imply that an alternative pathway(s) in which Akt act on p38 may exist, though low transfection efficiency may also contribute to incomplete blockade of ASK1 and MEKK3 activity and subsequent p38 activation in the Akt (-/-) cells.

P38 inactivation is associated with Akt activation in human cancer. The above results suggest that Akt acts upstream of p38 and blocks p38 activation. Because activation of Akt is a common phenomenon in different types of human cancers, we asked if p38 inactivation was also a common phenomenon in human cancer cells and correlates with Akt activation. To test if p38 inactivation was accompanied with Akt activation in human tumor tissues in vivo, we utilized tissue array slides to screen phospho-p38 and phospho-Akt expression in tumor tissues of different origins and normal or parallel normal organ tissues. We found that the phospho-Akt level was dramatically higher, while phospho-p38 was undetectable in most of the cancer tissues obtained from different types of solid tumors, such as cancers from breast, lung, liver, bile duct, gastric, colorectal, renal cell, ovarian, and uterine cancer; and, malignant lymphoma; and Schwannoma. In contrast, the intensity of phospho-p38 protein staining was relatively strong, while that of phospho-Akt staining was very weak in normal organs and parallel normal tissues. Representative data on the expression of phospho-p38 and phospho-Akt in normal versus tumor tissues obtained from the breast, lung, liver, and biliary duct are shown in Fig. 6A. By screening a panel of breast, ovarian, prostate, pancreatic, and colorectal cancer cell lines with phospho-specific antibody against p38 or Akt, we also observed a correlation between enhanced Akt phosphorylation and reduced p38 phosphorylation in these human cancer cell lines (data not shown). These data

support that p38 activity is repressed in different types of human cancer, which is associated with enhanced Akt activation.

To test if repression of p38 and activation of Akt also correlate with tumor stage in human cancer, we analyzed 10 normal breast tissue samples (including two normal nipple and eight normal breast tissues) and 50 cases of breast cancer at different stages in Histo-Array slides. These include 4 cases at stage I (T1), 21 cases at stage II (T2), 20 cases at stage III (T3) and 5 cases at stage IV (T4). Among the twenty-five cases of early stage tumors (T1 and T2), 12% of them are negative, and half of them are weakly positive for phospho-Akt staining. While in the advanced late stage tumor samples, all of them are positive and more than eighty percent of them are moderately to strongly positive for phospho-Akt staining (Fig. 6B), whereas the relative intensity of phospho-p38 staining was inversely correlated with the tumor stage (Fig. 6B). In the early stage tumor samples, only one third of them (36%) are positive for phospho-p38 staining and only one out of twenty-five cases are moderately positive for phospho-p38. In the late stage tumor samples (T3 and T4), eighty percent of them are negative for phospho-p38 staining, the rest are weakly positive for phospho-p38 staining (Fig. 6B). Comparing the phospho-p38 staining and phospho-Akt staining in the advanced late stage tumor samples, we observed an inverse correlation between the intensity of strong phospho-Akt staining versus weak phospho-p38 staining ($P < 0.0001$, Fig. 6C). Because phosphorylated p38 could be detected in most of the normal organ tissues but not in most of the cancer tissues or cell lines, indicating that p38 inactivation is also a common event in human cancer cells with Akt activation.

CR2 domain of E1A is required for downregulation of Akt phosphorylation and chemosensitization. The above results suggested that Akt regulated p38 activation in both physiological and pathological conditions, which indicates that E1A–mediated downregulation of Akt activity and upregulation of p38 activity are accompanied events, i.e., by repression of Akt activation, E1A enhanced p38 activity and sensitized cells to drug-induced apoptosis. To lay further genetic support for these conclusions, we proposed to map the domain(s) of E1A that is responsible for downregulation of Akt activity and demonstrate that the same domain is also critical for E1A–mediated upregulation of p38 and sensitization to drug–induced apoptosis. It is known that among the three conserved domains (CR) of E1A, CR1 and CR2 are associated with E1A–mediated sensitization to apoptosis (46). Therefore, we established E1A functional domain deletion mutation stable cells in MDA-MB-231, including wild type E1A, deletion mutation of CR1 (ΔCR1), and CR2 (ΔCR2) (Fig. 7A). We found that deletion mutation of the CR2 domain dramatically disrupted E1A's ability to downregulate Akt kinase activity and phosphorylation, abrogated E1A–mediated upregulation of p38 kinase activity and phosphorylation, and remarkably repressed E1A–mediated sensitization to Taxol–induced apoptosis (Fig. 7B and C), while the CR1 domain mutant only slightly affected E1A–mediated chemosensitization and downregulation of Akt and upregulation of p38 activities (Fig. 7 B and C). These results indicate that the same CR2 domain required for downregulation of Akt is also required for upregulation of p38 and sensitization to drug–induced apoptosis and thus supports the conclusions that Akt represses p38 activity and E1A by downregulation of Akt activity enhanced p38 activation and sensitized cells to anti-cancer drug–induced apoptosis.

DISCUSSION

The current study shows that activity of p38 is regulated by Akt and is deregulated partly due to Akt activation in human cancer. Activation of Akt antagonizes p38 activation, while inactivation of Akt results in p38 activation. And the adenoviral protein E1A by downregulation of Akt activity enhanced p38 activation and sensitized cells to apoptosis induced by different apoptotic stimuli. It is known that p38 participates in the regulation of apoptotic cell death through transcriptional upregulation of pro-apoptotic gene expression such as Fas ligand (11, 13, 20, 38). P38 is also involved in negative regulation of cell growth, as it represses cyclin D1 expression, it regulates G2-M transition through the regulation of cdc25 protein phosphatase and p53 protein (6, 42). Recently, inactivation of the p38 has been shown to contribute to the development of human cancers by suppressing p53 activation (7), suggesting a tumor suppressive function of p38. In contrast, Akt is known to upregulate the cyclin D1 expression while repressing Fas ligand expression and p53 stabilization (39, 50). Akt is also involved in regulation of G2-M transition (26, 40, 47). Thus, Akt may functionally antagonize the p38 effect on cellular processes ranging from cell cycle progression to cell death, though some cell types may respond differently (3, 8, 17, 19, 44). However, the regulation between Akt and p38 pathways is still unclear in the literature, and the current study provides a link between activation of Akt and inactivation of p38. As discussed above, Akt positively regulates cell growth but negatively regulates cell death, while p38 positively regulates cell death but negatively regulates cell growth. Given the results we obtained in Akt (–/–) MEF cells (Fig. 5B, and D) and the fact that Akt directly phosphorylates and negatively regulates the activation of ASK1 and MEKK3, the

upstream kinase of p38 (17, 28), we propose that Akt may repress p38 activation through the phosphorylation and inactivation of ASK1 or MEKK3 and, inactivation of Akt may result in p38 activation through the release of ASK1 and/or MEKK3 activity. Because either DN-ASK1 or DN-MEKK3 sufficiently repressed enhanced p38 phosphorylation in Akt1 (–/–) MEF cells (Fig. 5D), it also suggests that both ASK1 and MEKK3 are involved in Akt-mediated inactivation of p38. Regulation of p38 activity by Akt2 through ASK1 was also demonstrated recently by Yuan et al in their report on cisplatin-induced apoptosis (57), suggesting that both Akt1 and Akt2 may use a similar mechanism to inactivate p38.

We have shown that activation of p38 follows inactivation of Akt (Fig. 2 and Fig. 4B-D) when cells underwent apoptosis, suggesting that the pro- and anti-apoptotic signals may integrate each other to prepare cells to commit suicide. The relative Akt and p38 activity may determine a cell's response to apoptotic stimuli, as they can be observed in E1A-mediated sensitization to apoptosis induced by serum starvation, TNF-α, UV-irradiation and different categories of chemotherapeutic drugs (Fig. 4B-D). Expression of E1A may shift the balance of the pro- and anti-apoptotic signals by repressing Akt activity and enhancing p38 activity and thereby, favor the pro-apoptotic signal. Additional approaches could also be used by E1A to shift the intracellular signal integration to favor a pro-apoptotic signal, such as activation of p53 and caspase proenzymes, but these pathways may not contribute to the current study. For example, p53 and p14ARF were deleted in MDA-MB-231 cells and we did not detect any change in the expression level of p53 or p14ARF in E1A versus parental control cells (data not shown), i.e., the p53-dependent mechanisms may not contribute to E1A-mediated

sensitization to apoptosis in the current study. Although expression of E1A by infection of cells with either retroviral or adenoviral vector resulted in the accumulation of caspase proenzymes, such as caspase-3, -7, -8, and –9, by a direct transcriptional mechanism through enforced E2F-1 release in normal diploid human fibroblasts (IMR90)(37). We did not observe a consistent increase of these caspase proenzymes in the E1A stable cells established in human cancer cells with epithelial origin (Fig. 1D). In addition, sensitization to the DNA damage agent adriamycin–induced apoptosis by E1A is dependent on p53 status in normal fibroblast cells (37), while E1A dramatically sensitized adriamycin therapeutic effect in ovarian cancer SKOV3.ip1(5) and breast cancer MDA-MB-231 cells (Fig. 4A), which do not express functional p53. The discrepancy between normal diploid fibroblast and epithelial carcinoma cells in E1A–mediated sensitization to apoptosis may reflect the nature of the intrinsic difference between normal fibroblast and carcinoma cells. However, downregulation of Akt activity by E1A was also observed in E1A-mediated sensitization to cisplatin in human normal fibroblast IMR90 cells (54). Thus, targeting the key oncogenic survival factor Akt may represent a critical mechanism for E1A-mediated sensitization to anti-cancer drug–induced apoptosis in human cancer cells and normal fibroblast cells as well.

E1A has been shown to facilitate cytochrome *c* release from the mitochondria, which also contributes to E1A-mediated sensitization to anticancer drugs. However, the mechanism by which E1A facilitates cytochrome *c* release is unclear (14). Although we did not test whether E1A expression affected Bax translocation, which may also facilitate cytochrome c release, E1A expression or treatment with Taxol did not affect the levels of Bax protein in our system (Fig. 2A). Akt is known to play an important role in

maintaining mitochondria integrity and inhibiting the release of cytochrome *c* (11, 27). Overexpression of Akt confers resistance to Taxol by inhibiting Taxol-induced cytochrome *c* release (41). However, p38 is also involved in regulation of cytochrome *c* release (2). Therefore, it is possible that E1A may alter mitochondrial potential by downregulating Akt and upregulating p38, thereby facilitating the release of cytochrome *c* upon treatment with chemotherapeutic drugs, such as Taxol. Thus, downregulation of key survival factor Akt activity and subsequent upregulation of a pro-apoptotic factor p38 activity by E1A may constitute a fundamental approach for E1A-mediated sensitization to apoptosis.

The mechanisms underlying E1A-mediated downregulation of Akt activity is not yet clear. Obviously, E1A mediated downregulation of Her-2/neu and/or Axl may contribute to reduced Akt activation, as activation of either Her-2/neu or Axl leads to PI3K-Akt kinase activation and downregulation of Her-2/neu and/or Axl also contribute to E1A–mediated sensitization to apoptosis (21, 29, 30, 58). However, downregulation of Akt activity by E1A may not necessarily depend on E1A–mediated downregulation of Her-2/neu and/or Axl, because both MDA-MB-231 and MCF-7 cells are low Her-2/neu expressing cells and MCF-7 cells have undetectable expression level of Axl (35). In addition, deletion mutation of CR2 domain affects E1A–mediated downregulation of Akt (Fig. 7B), but it has no effect on E1A–mediated transcriptional repression of Her-2/neu (9). Thus, in addition to the Her-2/neu-dependent pathway, a Her-2/neu-independent pathway must exist for E1A to mediate downregulation of Akt activity leading to sensitization to apoptosis. In addition, overexpression of Her-2/neu or activation of Akt also leads to p53 destabilization (50, 59), suggesting that downregulation of Akt activity

by E1A may constitute an alternative pathway for stabilization of p53. Like Her-2/neu and p53, Akt also plays a critical role in the regulation of apoptotic cell death and the development of human cancer. Therefore, E1A–mediated downregulation of Akt and upregulation of p38 activities may have general implications for E1A–mediated tumor suppression and sensitization to apoptosis.

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Figure Legends

FIG. 1. Upregulation of p38 activity and downregulation of Akt activity by E1A correlated with E1A-mediated sensitization to Taxol-induced apoptosis. (A) Phospho-p38 (p38-p) and phospho-Akt (Akt-p) levels in E1A-expressing cells versus those in vector-transfected MDA-MB-231 and MCF-7 cells as shown. Total p38 (p38) and Akt (Akt) were used as loading controls. Kinase assay of p38 (B) and Akt (C) and densitometric analysis of relative p38 activity using GST-ATF-2 fusion protein as a substrate and Akt activity using GST-GSK-3-β fusion protein as a substrate in E1A-expressing cells versus vector-transfected control cells. **E**: E1A-expressing cells, **V**: vector transfected control cells. (D) Expression of caspase–3, –7, –8, and –9 proenzymes in E1A stable cells established in human breast cancer cell lines MCF-7, MDA-MB-231, MDA-MB-453, and ovarian cancer cell line 2774. **V**: vector control; **E**: E1A stable cells.

FIG. 2. Upregulated p38 activity and downregulated Akt activity correlate with E1A–mediated sensitization to Taxol–induced apoptosis. (A) Kinetics of PARP, Akt, p38, Bcl-2, p53, and Bax protein expression in 231-E1A cells before and after exposure to 0.01 μM Taxol. Bcl-2 phosphorylation and PARP cleavage were detected at 6 hours (hr) and 12 hr after exposure to Taxol and became more obvious thereafter. Phosphorylation of p38 was first detected after 30 minutes~2 hr, then become more obvious after 4hr treatment. Dephosphorylation of Akt could be detected even earlier at 5 minutes post-treatment. Actin was used as a loading control. A dramatic alteration of each molecular was marked with "*". (B) Dose-dependent effect of PARP cleavage and

Akt and p38 phosphorylation in MDA-MB-231 cells after exposure to Taxol for 24 hours. The doses of Taxol used ranged from 0, 0.001, 0.01, to 0.1 μM

FIG. 3. Activation of p38 and inactivation of Akt are required for E1A-mediated sensitization to Taxol-induced apoptosis. (A) Dose dependent effect of SB203580 on p38 phosphorylation in 231-E1A cells. (B) An isopropyl-β-D-thiogalacto-pyranoside (IPTG)-inducible, Flag-tagged dominant negative p38 (DN-p38) stable cell clone was established in 231-E1A cells. Repression of p38 activity by IPTG-inducible DN-p38 enhances Akt phosphorylation and abrogates E1A-mediated sensitization to Taxol in E1A-expressing cells in the presence of 5 μM IPTG for 24 hrs. (C) Western blot analysis of p38 and Akt in E1A expressing MDA-MB-231 cells and luciferase assay. The viability of cells with (lanes, 2,4,6) or without (lanes, 1,3,5) exposure to Taxol was measured using a luciferase assay. The pcDNA3-Luc vector was co-transfected into 231-E1A cells with (lanes 5 and 6) or without a hemagglutinin A (HA)-tagged, myristoylated, membrane-targeted constitutively active Akt construct (CA-Akt) (lanes 1-4) before treatment with Taxol. After exposure to Taxol for 4 hr, a portion of cells was harvested for protein extraction, while the rest were grown for 24 hr. In the absence of 20 μM SB203580, the level of phosphorylated p38 increased after exposure to Taxol (lanes 1–2). Expression of CA-Akt was detected using an anti-HA monoclonal antibody (lanes 5 and 6). (D) A portion of the above treated cells was also subjected to FACS analysis to measure apoptosis.

FIG. 4. Activation of p38 and inactivation of Akt represent a general cellular mechanism in response to different apoptotic stimuli. (A) Percentage of viable cells in vector-transfected (231-Vect) and E1A-expressing MDA-MB-231 cells (231-E1A) after exposure to different doses of adriamycin (ADR; 0.1, 1.0, and 10 μM), cisplatin (CDDP;

0.2, 2, and 10 μg/ml), gemcitabine (GEM; 0.2, 2, and 10 μg/ml), and methotrexate (MTX; 0.2, 2, and 10 μM) for 24 hr. Cell viability was measured using MTT assay. (B) Down-regulation of Akt activation and up-regulation of p38 activation correlated with drug-induced PARP cleavage in 231-Vect (V) and 231-E1A (E) cells. The doses used were 1 μM ADR, 2 μg/ml CDDP, 2 μg/ml GEM, and 2 μM MTX. (C) The doses of GEM and ADR used in MDA-MB-231 and MCF-7 cells were 20μg/ml and 20μM, respectively. (D) Down-regulation of Akt and up-regulation of p38 phosphorylation correlated with PARP cleavage induced by serum starvation, TNF-α, and UV-irradiation. 231-E1A cells were serum–starved, exposed to TNF-α (5 ng/ml), or UV-irradiated (6 J/cm2), while parental MDA-MB-231 cells were exposed to 10 times higher doses of TNF-α (50 ng/ml) and UV radiation (60 J/cm2). Both the attached cells and cells in suspension were collected, if not specified. W: whole cell lysate with both attached and suspended cells. **A**: attached cells only. **D**: detached or floated apoptotic cells.

FIG. 5. Physiological regulation of Akt and p38 pathways. (A) Stable E1A-expressing or parental MDA-MB-231 cells were serum-starved for 24 hr before exposure to 20.0 μM of 0.1 μM of Wortmannin. (B) Expression of phospho-p38 and phospho-Akt in Akt1 knockout MEF cells and myr-Akt–transfected Rat1cells. (C) CA-Akt was transiently transfected into both MDA-MB-231 and 293T cells. The cells were lysed after transfection, and Akt was immunoprecipitated. Western blot analysis of Akt, ASK1, and p38 interaction in 293T cells and MDA-MB-231 cells were performed. (D) Akt (–/–) MEF cells were grown in 6-well plate for 24 hrs and then transiently transfected by FuGENE 6 liposome (Cat. No. 1 814 443, Roche Molecular Biochemicals, Indianapolis, IN) at 3:1 ratio with either HA-tagged dominant negative ASK1 (DN-ASK1) or HA-

tagged dominant negative MEKK3 (DN-MEKK3) cDNA at the amount of 1μg and 10 μg, respectively. Cells were grown for another 36 hours and were then harvested and analyzed for p38 kinase activity using GST-ATF-2 fusion protein as a substrate. The expressions of phospho-p38, total p38, and HA-tag were also detected by respective antibodies.

FIG. 6. Inactivation of p38 associated with Akt activation in human cancer. (A) Immunohistochemical staining of phospho-p38 and phospho-Akt in different types of human cancer tissues in the Histo-Array slides. **Ca**, carcinoma. **ID Ca**, infiltrating ductal carcinoma. **BA Ca**, bronchioloalveolar carcinoma. **HC Ca**, Hepatocellular carcinoma. (B) The intensity of phospho-Akt and phospho-p38 stained in early and late stages of breast cancer. The staining intensity of phospho-Akt is significantly greater in late stage (stage III and IV) breast cancer, while positive phospho-p38 staining is predominantly in normal breast epithelial cells and early stage (stage I and II) breast cancer (both $P <$ 0.001). (C) Inverse correlation between phospho-Akt and phospho-p38 staining in late stage breast cancer tissue samples.

FIG. 7. CR2 domain of E1A is required for down-regulation of Akt and sensitization to drug–induced apoptosis. (A) A domain structure and map for deletion mutation of CR1 and CR2. (B) Akt and p38 kinase activity were measured and western blot analysis of PARP cleavage, phospho-Akt, phospho-p38, or E1A expression was performed in different domain deletion mutant stable cells. Actin was used as a loading control. Again, GST-GSK3-β and GST-ATF-2 fusion proteins were used as a kinase substrate for Akt and p38, respectively. (C) FACS analysis of apoptosis of wild-type E1A and different

domain deletion mutants E1A stable cells with or without treatment with 0.01 μM of Taxol for 21 hrs.

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Figure 1

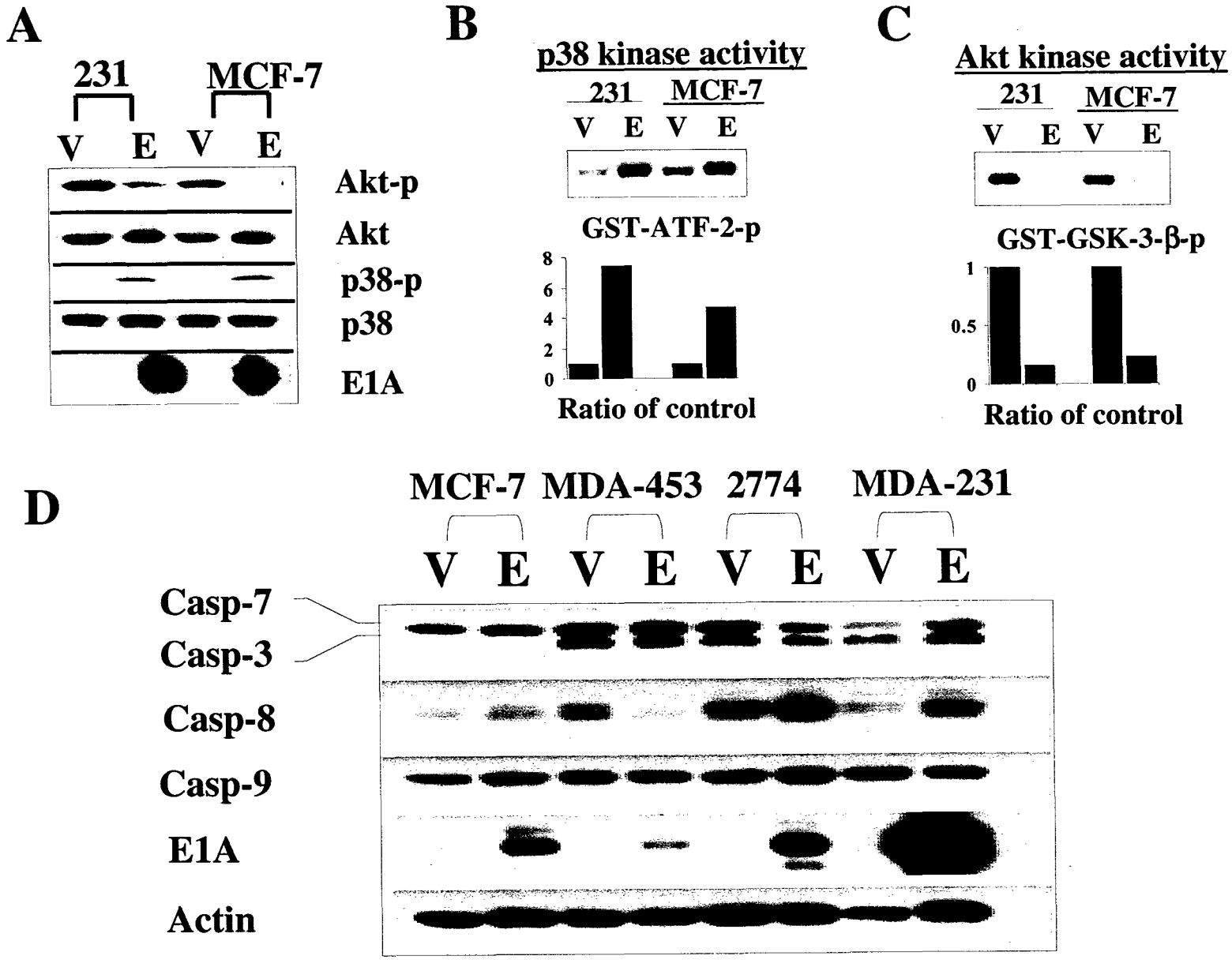
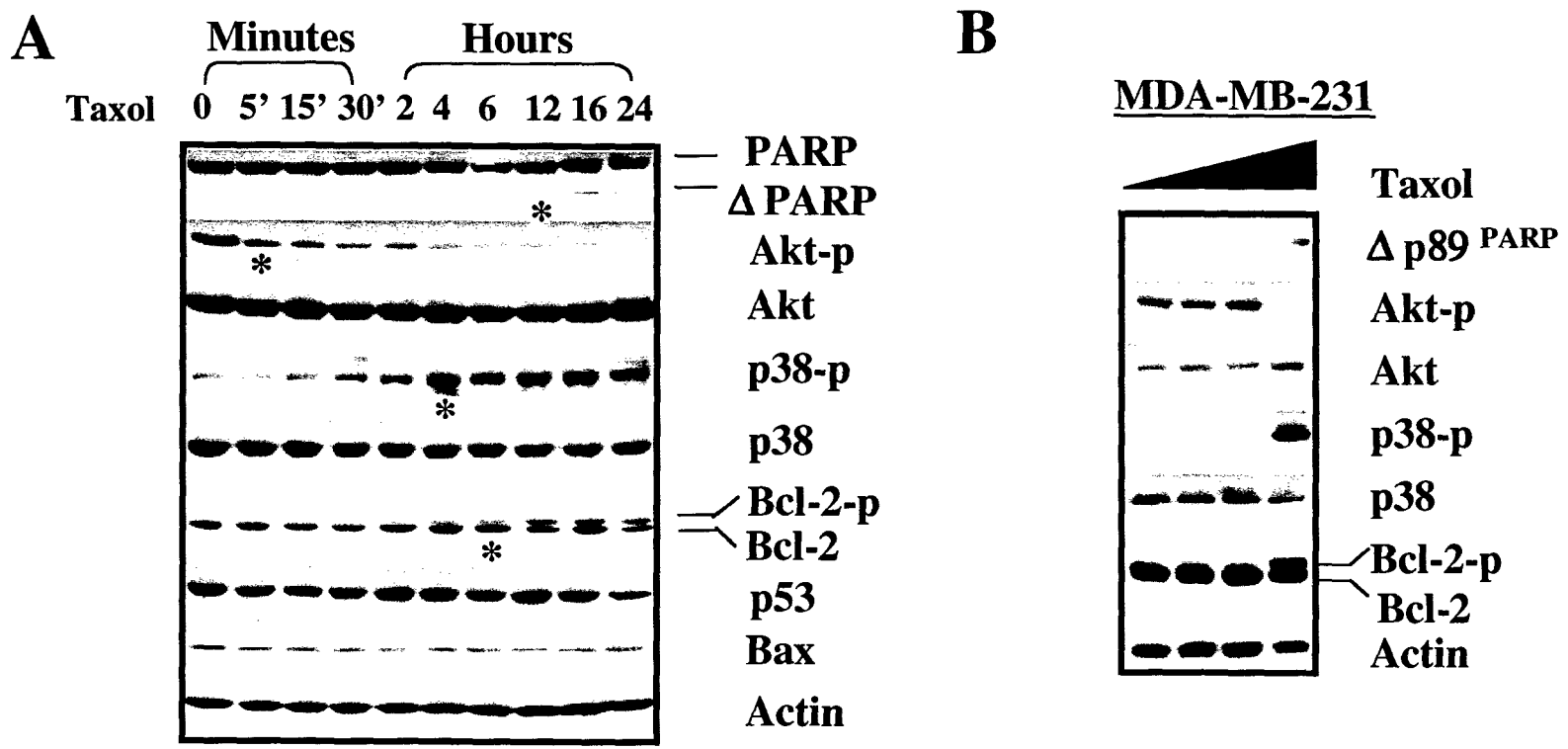

A
231
MCF-7
V E V E
Akt-p
Akt
p38-p
p38
E1A
B
p38 kinase activity
231 MCF-7
V E V E
GST-ATF-2-p
8
6
4
2
0
Ratio of control
C
Akt kinase activity
231 MCF-7
V E V E
GST-GSK-3-β-p
1
0.5
0
Ratio of control
D
MCF-7 MDA-453 2774 MDA-231
V E V E V E V E
Casp-7
Casp-3
Casp-8
Casp-9
E1A
Actin

Figure 2


A
Minutes
Hours
Taxol
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PARP
Δ PARP
Akt-p
Akt
p38-p
p38
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Bcl-2
p53
Bax
Actin
B
MDA-MB-231
Taxol
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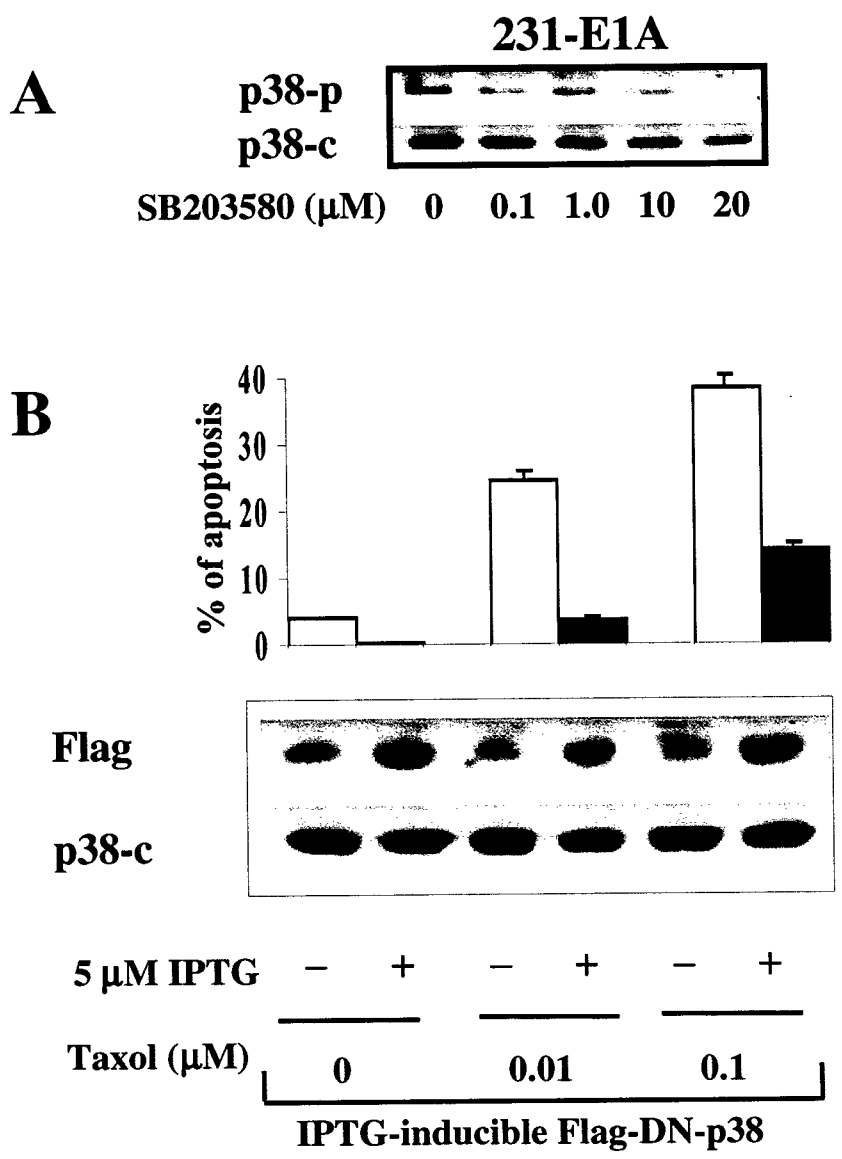
Akt
p38-p
p38
Bcl-2-p
Bcl-2
Actin

Figure 3


A
231-E1A
p38-p
p38-c
SB203580 (μM) 0 0.1 1.0 10 20
B
% of apoptosis
40
30
20
10
0
Flag
p38-c
5 μM IPTG – + – + – +
Taxol (μM) 0 0.01 0.1
IPTG-inducible Flag-DN-p38
C
Luc activity (% of control)
200
150
100
50
0
1 2 3 4 5 6
Akt-p
Akt-c
p38-p
p38-c
Anti-HA
D
% of apoptosis
27.5
17.5
7.5
-2.5
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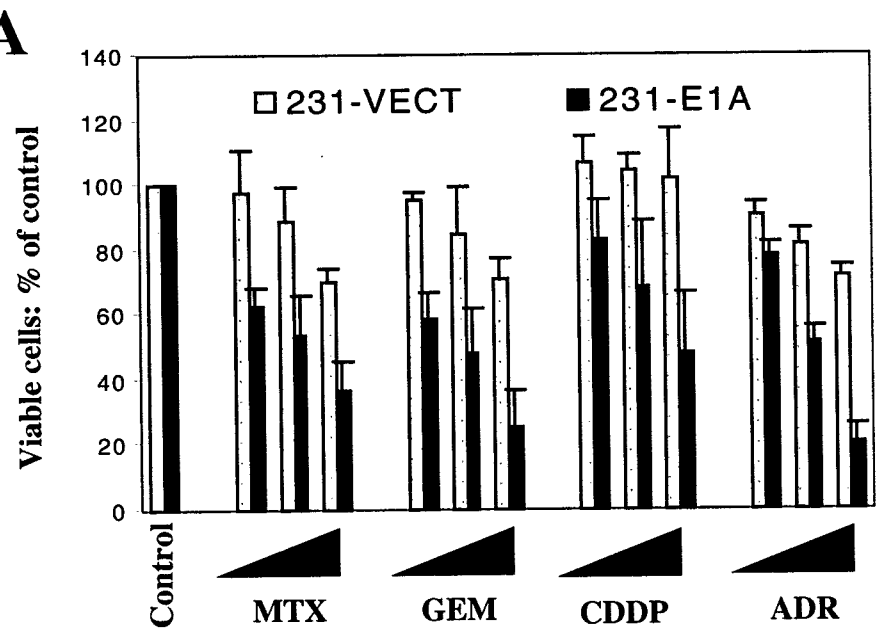
Taxol – + – + – +
SB203580 – – + + – –
CA-Akt – – – – + +

Figure 4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Viable cells: % of control
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100
80
60
40
20
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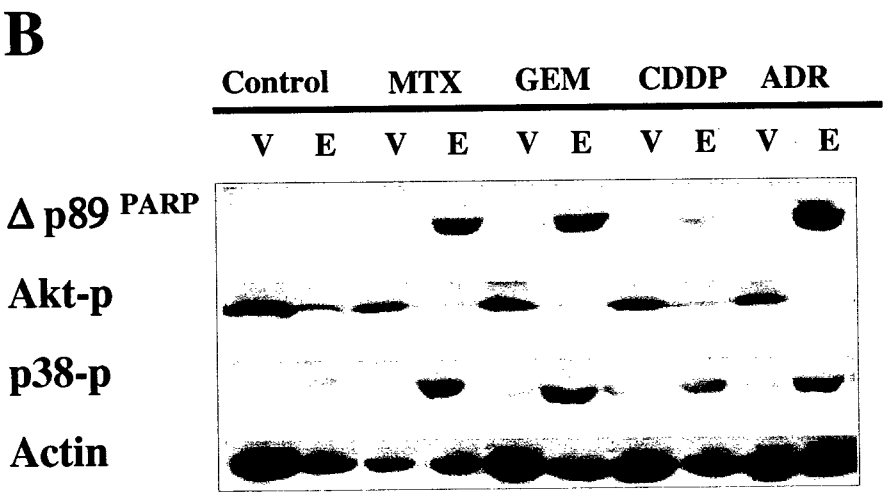
231-E1A
Control
MTX
GEM
CDDP
ADR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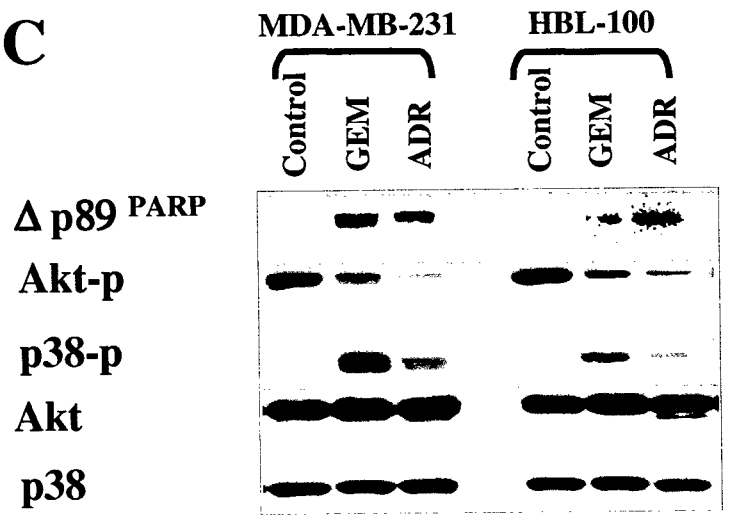
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GEM
ADR
Control
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Δ p89 PARP
Akt-p
p38-p
Akt
p38

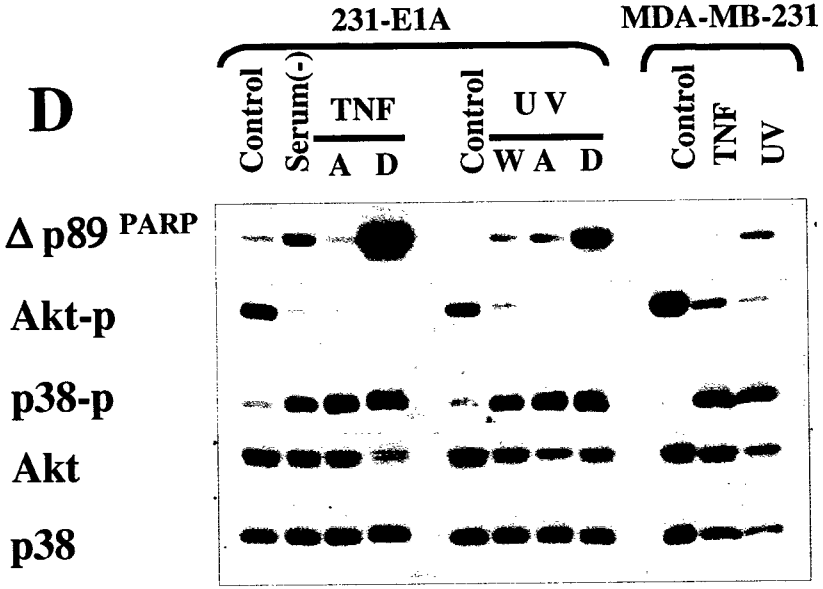

D
231-E1A
MDA-MB-231
Control
Serum(-)
TNF
A D
Control
UV
W A D
Control
TNF
UV
Δ p89 PARP
Akt-p
p38-p
Akt
p38

Figure 5

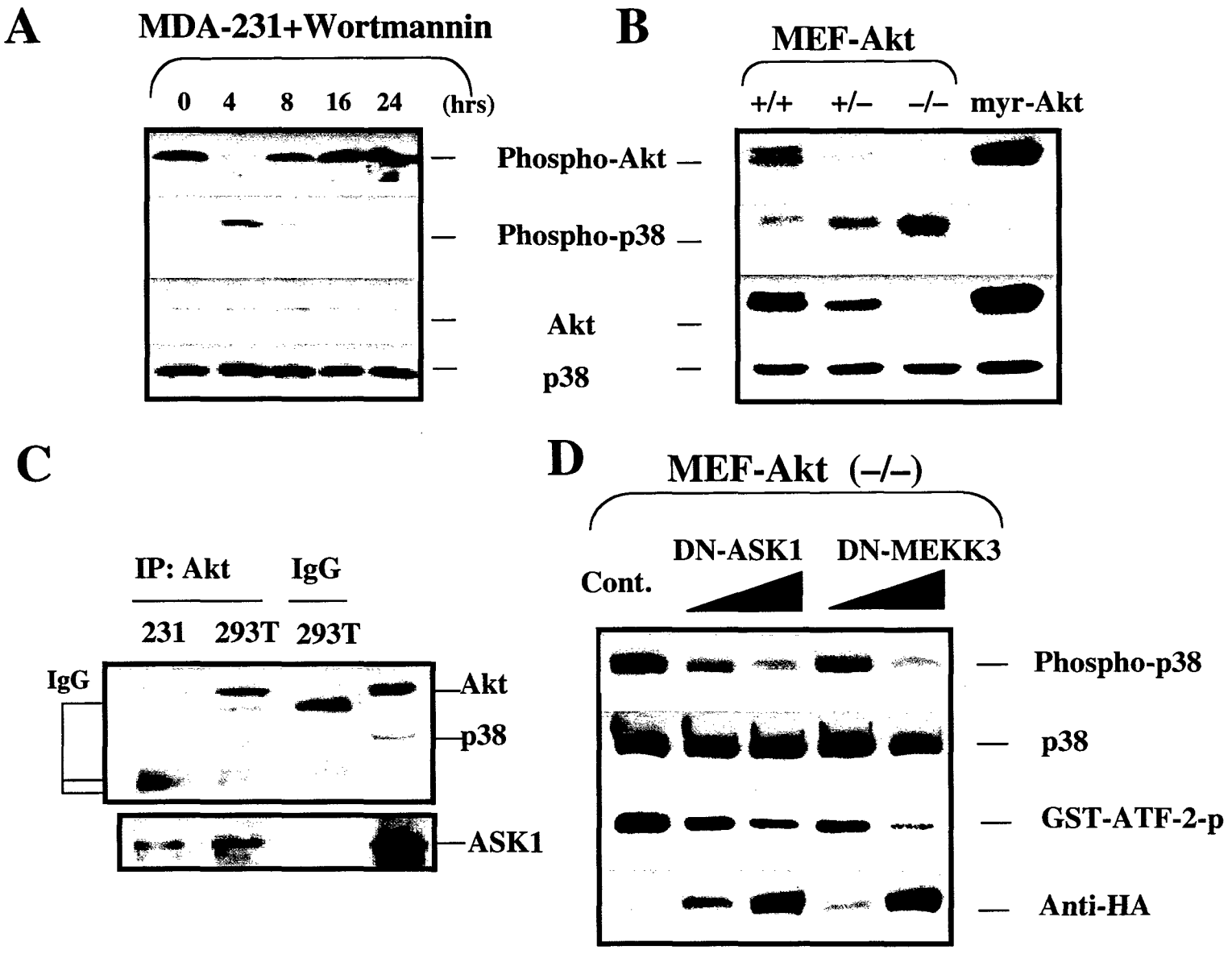
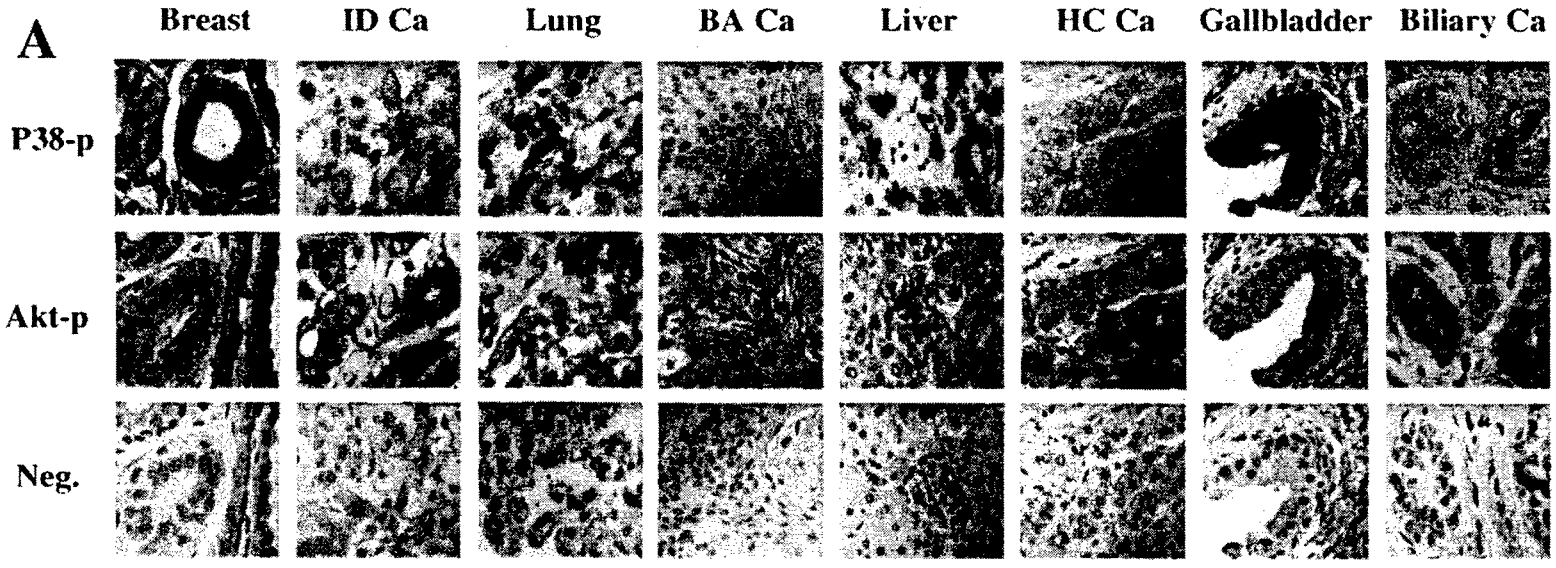

A
MDA-231+Wortmannin
0 4 8 16 24 (hrs)
Phospho-Akt
Phospho-p38
Akt
p38
B
MEF-Akt
+/+ +/– –/– myr-Akt
C
IP: Akt IgG
231 293T 293T
IgG
Akt
p38
ASK1
D
MEF-Akt (–/–)
Cont.
DN-ASK1
DN-MEKK3
Phospho-p38
p38
GST-ATF-2-p
Anti-HA

Figure 6

A



B

Immunohistochemistry analysis of phospho-Akt and phospho-p38 expression in human breast cancer tissues

	Phospho-Akt		Phospho-p38	
T Stage	T1~T2 (n=25)	T3~T4 (n=25)	T1~T2 (n=25)	T3~T4 (n=25)
−	3 /25 (12%)	0 /25 (0)	18/25 (64%)	20/25 (80%)
+	11/25(44%)	4 /25 (16%)	8 /25 (32%)	5 /25 (20%)
++	6 /25 (24%)	11/25(44%)	1 /25 (4%)	
+++	6 /25 (24%)	10/25(40%)		

C

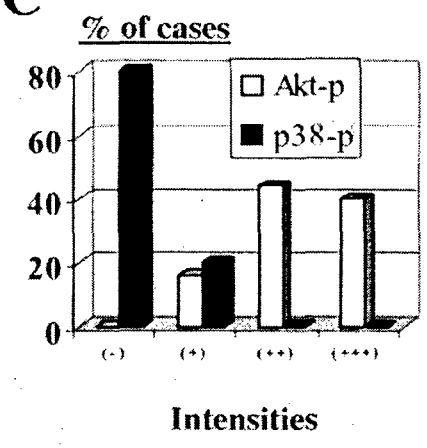
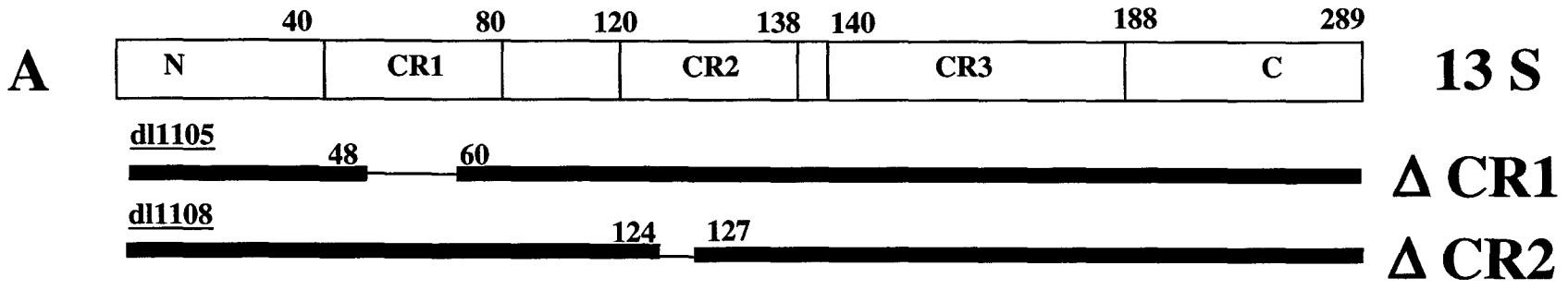
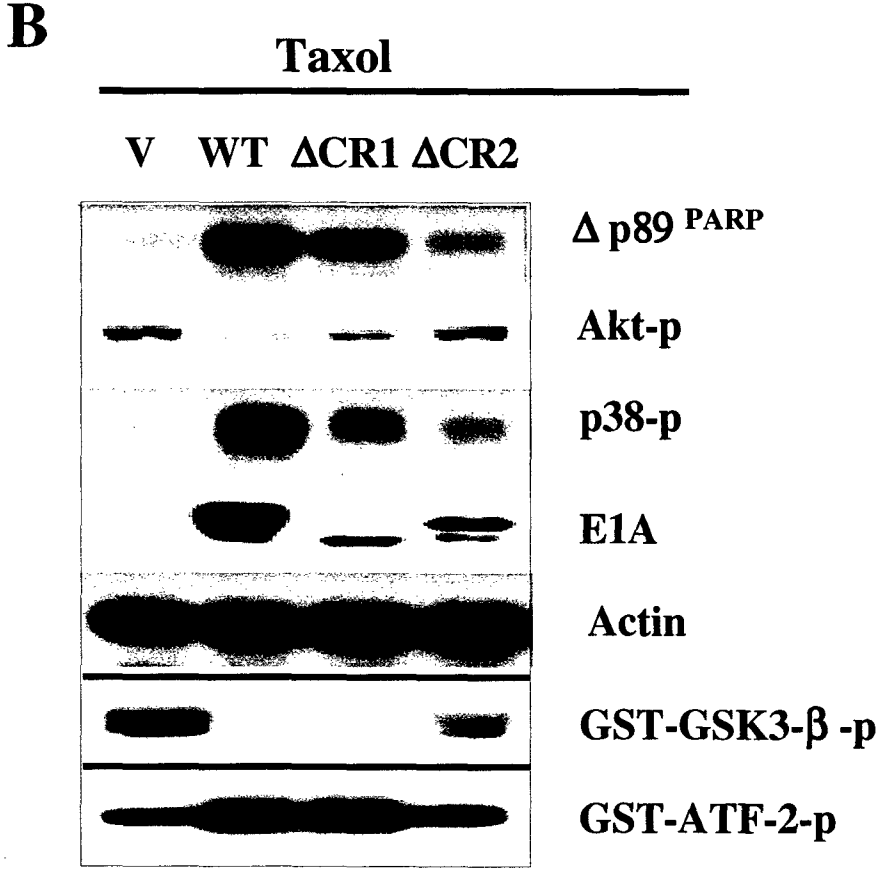
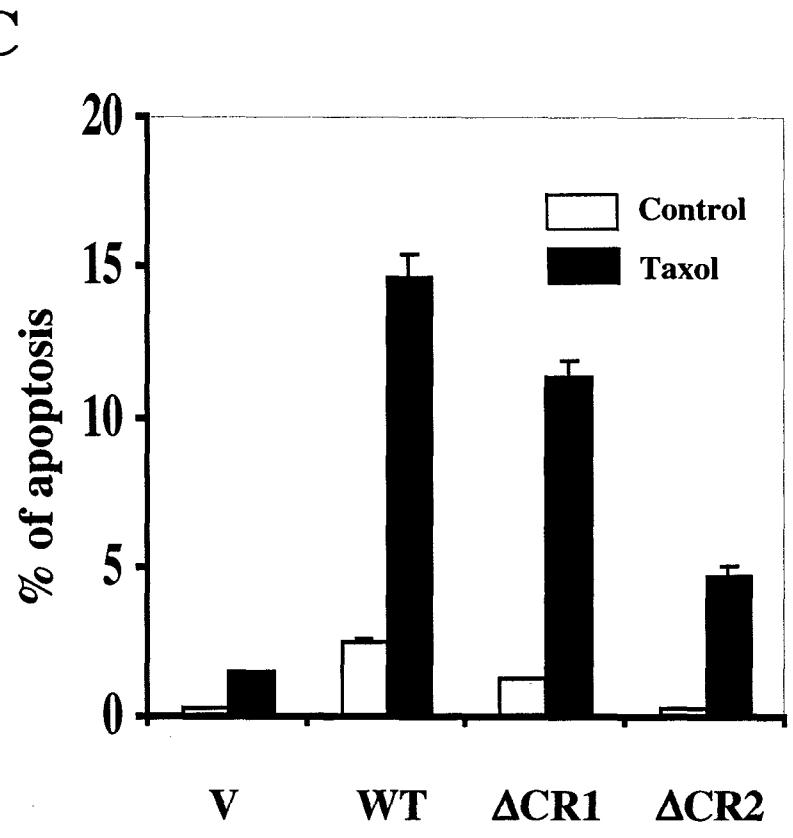


Figure 7


A
40
80
120
138
140
188
289
N
CR1
CR2
CR3
C
13 S
dl1105
48
60
Δ CR1
dl1108
124
127
Δ CR2


B
Taxol
V
WT
ΔCR1
ΔCR2
Δ p89 PARP
Akt-p
p38-p
E1A
Actin
GST-GSK3-β -p
GST-ATF-2-p


C
20
15
10
5
0
% of apoptosis
Control
Taxol
V
WT
ΔCR1
ΔCR2

Enhanced paclitaxel cytotoxicity and prolonged animal survival rate by non-viral mediated systemic delivery of E1A gene in orthotopic xonograft human breast cancer*.

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Running title: Enhanced therapeutic effect by systemic delivery of E1A gene

Keywords: breast cancer, xenograft, E1A, gene therapy, systemic gene delivery, non-viral delivery

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ABSTRACT

Paclitaxel is a promising frontline chemotherapeutic agent for the treatment of breast and ovarian cancers. In the Phase I clinical trial on E1A gene therapy, we observed that local administration of cationic liposome E1A complexes renders systemic detection of E1A mRNA expression in distant organs, suggesting that systemic gene delivery via intravenous injection may be feasible. Recently, we have developed a systemic nonviral delivery system by formulation of a new cationic liposome SN, which contains a surface-protection polymer to stabilize the liposome-DNA particles for i.v. injection. We wanted to determine if a systemic delivery of the adenovirial E1A gene by SN liposome could sensitize breast cancer cells to paclitaxel-induced killing. Therefore, we transfected E1A into these cells and then treated with paclitaxel *in vitro* and *in vivo*. In the *in vitro* study, the E1A-expressing MDA-MB-231 (231-E1A) and MCF-7 (MCF-7-E1A) cells were exposed to different doses of paclitaxel and the cellular cytotoxicities of paclitaxel were evaluated by MTT cytotoxicity assays, PARP cleavage, and FACS analysis. The results showed that expression of E1A enhanced *in vitro* paclitaxel cytotoxicity, as compared to the control cells. For the *in vivo* study, we first compared the therapeutic efficacy of paclitaxel between orthotopic tumor models established with vector-transfected MDA-MB-231 (231-Vect) versus 231-E1A stable cells, using tumor weight and apoptotic index (TUNEL assay) as the parameters. We found paclitaxel was more effective in shrinking tumors and inducing apoptosis in tumor models established with stable 231-E1A cells than the control 231-Vect cells. We then tested whether E1A could directly enhance paclitaxel-induced killing in nude mice, by using a

nonviral, surface-protected cationic liposome to delivery E1A gene via the mouse tail vein. We compared the therapeutic effects of E1A gene therapy with or without Taxol chemotherapy in the established orthotopic tumor model of animals inoculated with MDA-MB-231 cells, and found that a combination of systemic E1A gene therapy and paclitaxel chemotherapy significantly enhanced the therapeutic efficacy and dramatically repressed tumor growth ($P < 0.01$). In addition, survival rates were significantly higher in animals treated with combination therapy than in the therapeutic control groups (both $P < 0.0001$). Thus, the E1A gene therapy strategy may represent a novel and unique way to enhance the sensitivity of tumor cells to chemotherapy and, the current study provides the feasibility to extend the indications of E1A gene therapy trial to include low *Her-2/neu* expressing tumors, which may potentially benefit the majority of breast cancer patients with low *Her-2/neu* expression.

INTRODUCTION

Amplification and overexpression of *Her-2/neu* (or c-erbB-2) oncogene have been found in approximately 25% of human breast carcinomas and associated with poor prognosis (1). Using *Her-2/neu* as a target for the development of therapeutic agents proves to be an effective approach. For example Herceptin, a humanised monoclonal antibody against *Her-2/neu*, have demonstrated its efficacy by either Herceptin alone or in combination with cisplastin as a first-line therapy for the treatment of breast cancer patients with advanced disease whose tumors overexpressed *Her-2/neu* (2, 3). We have previously reported that the adenoviral type 5 E1A gene product also repressed *Her-2/neu* oncogene overexpression and suppressed the tumorigenic and metastatic potential of activated rat *Her-2/neu* oncogene transformed mouse 3T3 cells (4-9). Using either adenoviral vector or cationic liposome delivery system via local (intra-tumor, i.t.) or systemic administration of E1A gene (i.v.), we have also demonstrated its therapeutic efficacy in repressing tumor growth in nude mice carrying *Her-2/neu*-overexpressing tumors, such as cancers of breast, ovarian, and lung (10-17). Recently, Phase I clinical trials using liposome E1A gene therapy have been conducted in patients with recurrent breast and head and neck cancer and advanced cancers of the breast or ovary (18-21). The Phase I trials have provided the safety and feasibility for further trials and also proven the preclinical concept that down-regulation of Her-2/neu expression in *Her-2/neu*-overexpressing cancer cells by local administration of liposome E1A(18-20). However, unlike Herceptin, down-regulation of *Her-2/neu* oncogene is just one of the many anti-tumor functions mediated by E1A (22-25) and recent studies have shown that the tumor suppressor activity of E1A gene is also not limited to *Her-2/neu*-overexpressing tumors

(26, 27). In a number of cell lines, including a non-small lung carcinoma and breast cancer cell lines and rat melanoma cells that did not overexpress *Her-2/neu*, E1A was found to reduce the anchorage-independent growth in soft agar *in vitro* and tumor growth in nude mice (26, 27). In addition, expression of the E1A gene in stably transfected normal fibroblast and human cancer cells has also been shown to increase sensitivity to the *in vitro* cytotoxicity of several anticancer drugs, such as etoposide, cisplatin and paclitaxel (15, 16, 26, 28-33). Although normal fibroblast cells and some of the cancer cell lines do not overexpress *Her-2/neu*, expression of E1A also enhanced the cytotoxicity of anticancer drugs in these cells, suggesting that E1A-mediated sensitization to cytotoxic anticancer drugs may not necessarily depend on *Her-2/neu* status.

In the Phase I clinical trial on E1A gene therapy, we observed that local administration of cationic liposome E1A complex renders systemic detection of E1A mRNA expression in distant organs, suggesting that syetemic gene delivery via intravenous injection may be feasible in a nonviral cationic liposome delivery system (20). Recently, we have developed a systemic nonviral delivery system by formulation of a new cationic liposome SN, which contain a surface-protection polymer to stabilize the liposome-DNA particles for i.v. injection (34). The surface-protection SN formulation significantly enhanced the expression index of a therapeutic target gene such as the proapoptotic gene *bik* in the tumor tissue via i.v. delivery. In the current study, we utilized this SN formulation to delivery E1A gene by i.v. and tested if combination of E1A gene therapy with paclitaxel chemotherapy will enhance the cytotoxicity and anti-tumor effect of paclitaxel in orthotopic breast cancer xenograft established by inoculation of low *Her-2/neu* MDA-MB-231 cells in nude mice. We found that combination of

systemic E1A gene therapy with paclitaxel significantly enhanced paclitaxel-induced apoptosis and prolonged animal survival rates in the orthotopic model *in vivo*. Thus, the current study may extend the indications of future clinical trials of E1A gene therapy in combination with chemotherapy to include low *Her-2/neu* expressing tumors, which may potentially benefit the majority of breast cancer patients with low *Her-2/neu* expression.

MATERIALS AND METHODS

Cell lines. Human breast cancer MDA-MB-231 and MCF-7 cells were grown in Dulbecco's modified Eagle's medium/F-12 (Life Technologies Inc., Rockville, MD) supplemented with 10% fetal bovine serum. The stable E1A-expressing cell lines were established as previously described (35).

Paclitaxel. A stock solution of paclitaxel (Taxol, Bristol-Myers Squibb Co., Princeton, NJ) was stored at -80°C before use. For *in vitro* use, paclitaxel was diluted in serum-free medium at the required concentration. For *in vivo* use, paclitaxel was diluted in normal saline (NS) to a dose of 10 mg/kg in 150 μl per injection, once a week for 6 weeks.

Formulation. The gene delivery system, termed SN, was described by Zou et al (34). The DNA was entrapped in the SN-liposome using the thin-lipid film hydration method and extrusion through a filter with 0.2-μm-diameter pores (Gelman Sciences; Ann Arbor, MI) as described previously.

MTT assay. The metabolic conversion of tetrazolium salt (MTT) to formazan was used to indirectly measure the number of viable cells after exposure to Taxol. Cells (3×10^3/well) were seeded in triplicate in 96-well culture plates in 0.2 ml of culture

medium and allowed to adhere for 24 h. After exposure to Taxol for indicated time periods, 20 µl of MTT was added to each well. Cells were cultured for an additional 2 h, and 100 µl of extraction buffer (20% SDS in 50% *N,N*-dimethyl formamide, pH 4.7) was added to the culture medium. The cells were incubated overnight at 37 °C, and the plate absorbency was measured at 570 nm.

Propidium iodide staining and FACS analysis. Samples of 2 x 10^6 cells were collected, washed once with phosphate-buffered saline (PBS), and fixed with 70% ice-cold ethanol overnight. After fixation, cells were washed with PBS to remove residual ethanol, pelleted, and resuspended in PBS containing 50 µg/ml of propidium iodide (Sigma, St. Louis, WA). The staining was performed at 4 °C for at least 30 min, and samples were analyzed using a FACScan (Becton-Dickinson, San Jose, CA) in the core facility at The University of Texas M. D. Anderson Cancer Center.

Athymic Nude Mice. Four to 6-week-old female athymic BALB/c-*nu/nu* mice were purchased from Charles River Laboratories (Wilmington, MA). The animals were allowed to acclimate for 7 days before the study initiation. All of the animals were housed under pathogen-free conditions, and were given water and chow *ad libitum*. Animal care and use were in accordance with Institutional and NIH guidelines.

Orthotopic breast cancer model and systematic E1A gene therapy in nude mice. MDA-MB-231 cells (1 x 10^6 cells/0.1 ml) or 231-E1A cells (2 x 10^6 cells/0.1 ml) were subcutaneously injected into the mammary fat pad (m.f.p.) of female, athymic mice. Tumors were allowed to develop for 21 days and mice were then randomly grouped and treated with SN-liposome alone (SN, i.v.), Taxol alone (i.p., 10 mg/kg/injection, once a week for 6 weeks), E1A alone (i.v., 15 µg/mouse/injection, injection into mouse tail vein

twice a week for 6 weeks), or E1A (i.v., 15μg/mouse/injection) plus Taxol (i.p., 10 mg/kg/injection, 24 hours after E1A injection, for 6 weeks). Both maximum and minimum diameters of the resulting tumors were measured twice a week using a slide caliper. Tumor volumes were calculated by assuming a spherical shape and using the formula, volume = $4/3r^3$, where r = 1/2 of the mean tumor diameter measured in two dimensions. Mice were sacrificed when their tumors were larger than 2 cm in diameter otherwise mice will keep growing and subject for survival rate analysis.

Immunohistochemical Analysis. To evaluate expression of E1A in stable MDA-MB-231 cells, cells in exponential growing were harvested and washed with PBS twice and then cytospuned. Cytospun cells were fixed for 10 min in 4% formaldehyde (Sigma), washed with PBS, dried, and stored at -80°C or subject to immunohistochemical analysis. To evaluate expression of E1A protein level in tumor tissues in vivo, stable E1A expressing cells or control vector cells were inoculated into MPF of nude mice. After a palpable tumor was formed, approximately 4 to 6 weeks, tumor dissected and fixed in 10% formalin and embedded in paraffin blocks. Paraffin-embedded sections were then pretreated with dewax and rehydrated. They were washed in xylene and rehydrated through a graded series of ethanol and redistilled water. The paraffin sections or cytospun slides were incubated with E1A M73 monoclonal antibody (Oncogene Science, Inc., Cambridge, MA) diluted 1:20. The slides were then incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories) or biotinylated horse anti-mouse IgG (Vector Laboratories) diluted 1:200 in PBS. The slides were then incubated with an avidin-biotin-peroxidase complex (Vector Laboratories), and the peroxidase-catalyzed product was visualized with 0.125% aminoethyl carbazole chromogen buffer (Sigma Chemical Co.).

***In vivo* apoptotic (TUNEL) assay.** For *in vivo* apoptotic assay, tumors were fixed in 10% formalin and embedded in paraffin blocks as described above. Tissue sections were incubated with proteinase K (20 μg/ml in 10 mM Tris/HCl, pH 7.4 to 8.0, for 15 min at 37°C), permeabilized in 0.1% Triton-X-100 in 0.1% sodium citrate, and then labeled with the TUNEL reaction mixture (Boehringer Mannheim, Indianopolis, Indiana) according to the manufacturer's protocol. Briefly, biotinylated nucleotide mix and TdT enzyme were added and incubated for 1 h at 37°C; slides were washed in PBS, blocked in hydrogen peroxide, and incubated in streptavidin horseradish peroxidase. The slides were developed in 3,3'-diaminobenzidine and counterstained with hematoxylin. The apoptotic cells (brown staining) were counted under a microscope. The apoptosis index was defined by the percentage of brown cells among the total cells of each sample. Ten fields with >200 cells in each were randomly counted for each sample.

RESULTS

E1A-mediated sensitization to paclitaxel correlated with E1A-induced apoptosis *in vitro*. Paclitaxel is a promising frontline chemotherapeutic agent for treating breast and ovarian cancers. To test whether E1A can sensitize cells to paclitaxel-induced apoptosis in low *Her-2/neu* expressing cells in cell culture *in vitro*, we treated MDA-MB-231 (p53 mutant) and MCF-7 (p53 wild type) human breast cancer cell lines and their E1A-expressing cells with different dosages of paclitaxel and performed MTT cytotoxicity assays. We found that stable expression of wild-type E1A enhanced sensitivity to paclitaxel-induced killing in MDA-MB-231 cells in a dose dependent

manner. Compared with their IC50 dosage, the E1A-expressing stable cells were ten times more sensitive than that of the paclitaxel-treated parental or vector-transfected control cells (Figure 1A). Stable expression of E1A also enhanced the sensitivity of MCF-7 cells to paclitaxel, although to a lesser extent. Interestingly, a revertant of the MCF-7 clone that lost its E1A expression during long-term *in vitro* cell culture also lost its sensitivity to paclitaxel (Figure 1B). Three additional independent E1A stable clones from each cell line were analyzed, all of which expressed high levels of E1A protein and showed similar sensitivity to paclitaxel (data not shown).

To address whether apoptosis was involved in E1A-mediated sensitization to paclitaxel, we examined the presence of poly (ADP-ribose) polymerase (PARP) cleavage as an apoptotic cell death marker. We detected a dose-dependent increase of PARP cleavage in E1A-expressing MDA-MB-231 (231-E1A) and MCF-7 (MCF-7-E1A) cells upon treatment with 0.1 μM to 0.001 μM paclitaxel. In parental and 231-Vect cells, however, PARP cleavage was observed only upon treatment with 0.1 μM paclitaxel, while in the revertant MCF-7 cell clone, it was not detected (Insert, Figure 1A and B). Fluorescence-activated cell sorting (FACS) analysis also supported that E1A enhanced paclitaxel-induced apoptosis, as demonstrated by an increased proportion of sub-G1-phase cells in E1A-expressing cell lines (Figure 1C). Paclitaxel is well known to induce G2/M arrest (as was observed in parental or vector-transfected cells) (36), but we noticed that the expression of E1A altered paclitaxel-induced G2/M arrest by an as yet unknown mechanism. Thus, E1A-mediated sensitization to paclitaxel correlated with E1A-induced apoptosis in low Her-2/neu expressing breast cancer cells and was independent of p53 status.

Expression of E1A enhances paclitaxel-induced anti-tumor effect *in vivo*.

To test whether E1A also mediated sensitization to paclitaxel *in vivo*, we inoculated stable E1A-expressing (231-E1A) and vector-transfected (231-Vect) MDA-MB-231 cells into mouse MPF. In order to ensure tumor formation in 231-E1A inoculated animals, twice amount of 231-E1A cells were inoculated. When tumor established, animals were then treated with or without paclitaxel, via intra-peritoneal injection, once a week, for four consecutive weeks. Mice were grown for another six weeks and were then sacrificed. Tumor tissues were collected and measured. The mean tumor weight of animals inoculated with 231-E1A cells is significantly lower than that of animals inoculated with 231-Vect cells (Figure 2A, $P < 0.01$), even though the original inoculated cell number is higher in 231-E1A group. The result supported the previous reports that expression E1A alone had a significant effect on repression tumor growth *in vivo* independent of *Her-2/neu* status (26, 27). Treatment with paclitaxel of tumors formed by inoculation of 231-E1A cells achieved 80% reduction of tumor weight in comparison with untreated control 231-E1A tumors. Paclitaxel treatment of tumors formed by 231-Vect cells also achieved nearly 50% reduction of tumor weight. These results indicate that paclitaxel alone is sufficient to achieve a fifty-percent cytoreduction of tumor mass in compared with mice treated with normal saline (NS) (Figure 2A). Comparing the percentage of tumor shrinkage in paclitaxel treated 231-Vect tumor group, paclitaxel treatment of 231-E1A tumor achieved a better tumor shrinkage than that of the paclitaxel treated 231-Vect animals, i.e., the 231-E1A group was more sensitive to Taxol ($P < 0.01$). Thus, expression of E1A also sensitized paclitaxel cytotoxic effect in established tumors *in vivo*.

To test if E1A was still expressed in tumor tissue in vivo, we then analyzed E1A expression in tumor tissue slides and used original 231-E1A stable cells as the positive control by immunohistochemistry. Modest to strong E1A staining was detected in more than ninety percent of 231-E1A stable cells cultured *in vitro* in the cytospun slides (Figure 2B). Whereas, less than half of the cells were positive for E1A staining in tumor tissue samples obtained from the original 231-E1A inoculated animals, and most of them only had modest or weak staining of E1A (Figure 2B). This result suggests that most of the cells with higher E1A-expressing were lost under the *in vivo* selection pressure during tumor development. None of the control 231-Vect stable cells or 231-Vect inoculated tumors had a positive staining for E1A (Figure 2B). These results also imply that expression E1A repressed tumor growth in vivo while loss of E1A expression may result in tumor development in 231-E1A stable cells. Or in other words, only cells with relatively lower E1A expression survived the in vivo selection pressure and developed tumor. That is why the tumor formation in animals inoculated 231-E1A stable cells is slower and smaller than that of the animals inoculated with 231-Vect cells (Figure 2A). Even though 231-E1A inoculated tumors have modest to weak E1A expression, they are still more sensitive to paclitaxel than 231-Vect inoculated to tumors (Figure 2A), it implies that expression of higher level of E1A protein is not a prerequisite for E1A-mediated sensitization to paclitaxel *in vivo*. This makes an important point for the application E1A for a systemic gene therapy, as inducing higher level of target protein expression tends to be very difficult in a gene therapy setting.

E1A-mediated sensitization to paclitaxel correlated with E1A-induced apoptosis *in vivo*. To test if E1A-mediated sensitization to paclitaxel

cytotoxicity *in vivo* is also through apoptotic cell death, we used a deoxynucleotide transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay to compare apoptotic index in paclitaxel-treated tumor tissue samples obtained from animals inoculated with 231-E1A cells versus those from animals inoculated with 231-Vect cells. In order to detect apoptotic cells *in vivo* following paclitaxel treatment, tumor-bearing animals were treated with paclitaxel (15mg/kg/animal) once by i.p. injection. Animals were sacrificed 24 hours after paclitaxel treatment and tumor tissue samples were then collected and sectioned for TUNEL assay. In tumor tissues obtained from 231-E1A-inoculated animals (n=3), a few TUNEL-positive cells were detected without treatment with paclitaxel, however, treatment with paclitaxel remarkably increased TUNEL-positive cells (from 9% to 18% positive cells). Treatment with paclitaxel also enhanced TUNEL-positive cells in tumor tissue samples obtained from 231-Vect-inoculated animals (n=3) (from 3% without paclitaxel to 7% with paclitaxel) (Figure 3 A and B). Statistic analysis showed that paclitaxel-treated 231-E1A tumor tissues had a significantly higher percentage of TUNEL-positive cells than did paclitaxel-treated 231-Vect tumor tissues ($p < 0.001$). Again, the result suggests that expression of E1A significantly enhanced paclitaxel cytotoxicity in tumor tissue *in vivo*. The relatively higher frequency of TUNEL-positive cells detected in tumor tissues obtained from 231-E1A-inoculated animals also suggests that the relatively small tumors formed in 231-E1A-inoculated animals in Figure 2A may in part due to the enhanced apoptosis in 231-E1A tumors.

Enhancement of paclitaxel-induced anti-tumor effect by systemic delivery of E1A gene in orthotopic breast cancer model *in vivo*. To explore whether E1A

could directly enhance paclitaxel-induced killing *in vivo*, we designed systemic E1A gene therapy experiments in mice by intravenous (*i.v.*) injection of liposome encapsulated E1A gene via the mouse tail vein with or without combinaiton of paclitaxel chemotherapy. MDA-MB-231 cells were inoculated orthotopically into mouse MPF and were allowed to grow until a palpable tumor was formed. When tumors were established, animals were regrouped and randomly assigned to each experimental group. We used SN liposome formulation as our gene-delivery system because of its relatively high efficiency *in vivo* (34). SN liposome (SN) or SN liposome encapsulated E1A gene was given twice a week for six weeks through mouse tail vein, while paclitaxel were given once a week by intra-peritoneal injection for six weeks. We started to monitor tumor size once a week when animals received the first time of each treatment and continued until six weeks later after each treatment were ended (total for 12 weeks). We then compared the mean tumor volume between each treatment group in order to assess the therapeutic effects of each treatment regime. Compared with control SN liposome alone, treatment with either systemic delivery of E1A gene or *i.p.* injection of paclitaxel repressed tumor growth during the 12-week observation period ($P < 0.05$, $P < 0.01$) (Fig. 4). Compared with treatment of systemic E1A gene alone or paclitaxel alone, a combination of E1A gene therapy and paclitaxel chemotherapy produced significantly enhanced therapeutic effect of paclitaxel and dramatically repressed tumor growth (both $P < 0.0001$) (Fig. 4). In addition, tumors were completely eradicated in four out of seven mice treated with combination therapy. Thus, combination of systemic E1A gene therapy with paclitaxel chemotherapy had a synergistic effect in repression tumor growth in orthotopic breast cancer model *in vivo*.

Prolonged animal survival rate by non-viral mediated systemic delivery of E1A gene in combination with paclitaxel chemotherapy in orthotopic xonograft human breast cancer model. In order to see if combination of systemic E1A gene therapy with paclitaxel chemotherapy could increase animal survival time, we extended our observation on animals after receiving the above treatments. The end point criteria include either tumor size reached 2 cm in diameter or animals are moribund. In animals that received treatment with liposome alone, most of the animals were died or sacrificed (because their tumors are too big) within three months after treatment. Although E1A-liposome alone did not dramatically repress tumor growth as that of paclitaxel alone did (Figure 4), treatment with systemic delivery of E1A gene achieved a comparable enhancement than that of the animals treated with paclitaxel alone in terms of prolongation of the animal survival times ($P < 0.05$) (Figure 5). Compared with survival rate of animals with treatment of SN liposome, treatment with either E1A gene or paclitaxel had a longer survival time (both $P < 0.05$). In nearly a year's close observation, we found that combination of systemic E1A gene therapy with paclitaxel chemotherapy achieved a significantly better outcome in animal's survival rates, compared with all the three single treatment regimes, including SN-liposome alone, liposome-E1A, or paclitaxel (all $P < 0.01$) (Fig. 5). In addition, three out of seven animals treated with combination therapy achieved over one year's tumor-free survival. Thus, combination of systemic E1A gene therapy with paclitaxel chemotherapy significantly enhanced paclitaxel's antitumor effect and dramatically prolonged animal survival rates in the orthotopic breast cancer model *in vivo*.

DISSCUSION

In the current study, we showed that E1A mediated sensitization to the paclitaxel-induced apoptosis *in vitro* in low *Her-2/neu* expression breast cancer MCF-7 and MDA-MB-231 cells. We showed that expression of E1A also enhanced the therapeutic effect of paclitaxel in orthotopic breast cancer model *in vivo*. In addition, we demonstrated that combination of a systemic E1A gene therapy using a surface-protected SN-liposome E1A complex formulation with paclitaxel chemotherapy significantly enhanced paclitaxel's antitumor effect and dramatically prolonged animal survival rates in low *Her-2/neu* expression breast cancer MDA-MB-231 cell inoculated orthotopic breast cancer model *in vivo*. Our data showed that neither E1A gene therapy alone nor paclitaxel alone was as efficient as the combination of both E1A gene therapy and paclitaxel chemotherapy in terms of repression of tumor growth in vivo and prolongation of animal survival rate. This result suggested that a synergistic effect achieved in the combination group. In our previous reports, we failed to show a chemosensitization effect of E1A in low *Her-2/neu* expressing cells, such as MDA-MB-231 or MDA-MB-435 cells, by either adenoviral vector mediated delivery or DC-Chol cationic liposome (14, 15). Part of the reasons for this difference may come from the variance of delivery efficiency, although other reasons may also contribute to the difference and will be discussed later. Because MDA-MD-231 or MDA-MB-435 cells tend to have a lower infection efficiency than the *Her-2/neu* overexpression MDA-MB-453 or MDA-MB-361 cells in the up-taken of the adenoviral particles in cell culture *in vitro* (14, 15) and the DC-Chole liposome may not as efficient as SN formulation. As we reported recently, the modified cationic liposome SN formulation containing a surface-protection polymers, which entraps condensed DNA

into the internal aqueous phase of the liposomes, stabilize the liposome-DNA particles from the attack of serum components, and make it an efficient i.v. DNA delivery system (34). The current study first time showed that combination of a nonviral systemic E1A gene therapy with paclitaxel chemotherapy is effective for the treatment of *Her-2/neu* low expressing breast cancer cells in a pre-clinical systemic gene therapy setting. A similar synergistic mode of action was also observed in our recent study on a *Her-2/neu* overexpression breast cancer cell line MDA-MB-361 by using another lipopolyplexes LPD formulation to systemically delivery of E1A gene (17). Thus, these complimentary studies suggested the feasibility for a potential future clinical trial by using the combination of systemic E1A gene therapy with paclitaxel chemotherapy in patient with either *Her-2/neu* overexpression tumor or low Her-2/neu expression tumor, which represents the majority of the breast cancer patients. Unlike the trials on *Her-2/neu* overexpression tumors that used *Her-2/neu* expression as the marker, test on low *Her-2/neu* expression tumors may need a surrogate marker or markers in order to monitor if the therapy works in this subgroup of tumors. In the literature, previous studies on E1A-mediated sensitization to drug-induced apoptosis in different cell systems or cell free systems have identified a few other molecules that are also linked to E1A-mediated chemosensitization other than down-regulation of *Her-2/neu*, such as the pro-apoptotic protein Bax, Apaf-1, p19ARF, pro-caspase-2, -3, -7, -8, and –9, or an yet unidentified inhibitor that ordinary provides protection against cell death (16, 37-44). Whether one of these molecules or other unknown molecules could be used as a surrogate marker to monitor the therapeutic effect of this combination therapy in tumors with low *Her-2/neu* expression needs further assessment.

It is worthwhile mentioning the important clinical implications that the concentration of paclitaxel we tested in the *in vitro* study. Because the paclitaxel concentration that can kill cancer cells *in vitro* in the presence of E1A is clinically relevant. A plasma concentration of 5 μM to 10 μM paclitaxel can be achieved after bolus infusion of paclitaxel, but it rapidly falls to a level of several hundred nanomolar or less (36). Our experiments showed that 10 nM paclitaxel was sufficient to induce apoptosis *in vitro* in E1A-expressing cells but not in parental or vector control cells (Figure 1). This indicates that clinically relevant concentration (5~200 nM) of paclitaxel is sufficient to kill E1A-expressing cells, but parental cells require much higher dosage, which may be difficult to be achieved in a clinical setting (36). We have previously shown that *Her-2/neu* overexpressing cells are resistant to paclitaxel-induced apoptosis and E1A, through downregulation of *Her-2/neu*, can sensitize cellular response to paclitaxel-induced apoptosis (15, 16, 45, 46). In those studies, we could not detect E1A-mediated chemosensitization in the low *Her-2/neu*-expressing cells, such as breast cancer MDA-MB-435 cells. The major reason for this discrepancy was due to the paclitaxel concentrations tested. The *Her-2/neu*-overexpressing cancer cells are resistant to paclitaxel even at a dosage of 10.0 μM, in the presence of E1A, they became sensitive even at the dose of 1.0 μM of paclitaxel (15, 16). The low *Her-2/neu*-expressing cells, such as MDA-MB-231and MDA-MB-435, are much more sensitive to paclitaxel, even at paclitaxel concentration of 0.1 μM they are still sensitive (15). Therefore, in the previous studies, the dose of paclitaxel was too high to detect E1A-mediated paclitaxel sensitization that was found in the present study. Thus, the E1A gene therapy strategy may represent a novel and unique way to enhance the sensitivity of tumor cells to

chemotherapy and the current study provides solid pre-clinical data that may help in developing further clinical trials using the combination of chemotherapy with systemic gene therapy. In addition, it also extends the indications of E1A gene therapy trial to include low *Her-2/neu* expressing tumors, which may potentially benefit the majority of breast cancer patients with low *Her-2/neu* expression.

Legend to Figures

Figure 1. E1A–mediated sensitization to paclitaxel-induced apoptosis *in vitro*.

A: MTT assay. Percentage of viable cells after exposure to 0.1μM, 0.01 μM, and 0.001 μM of paclitaxel (Taxol) for 24 hr in MDA-MB-231 (231), vector-transfected cells (Vect), and E1A-expressing cells (E1A) detected using MTT assay. The number of viable cells without paclitaxel treatment was defined as 100%. The insert shows a cleaved PARP p89 fragment (ΔPARP) that was detected using a rabbit polyclonal antibody against cleaved PARP. Lanes 1, 5, and 9 in the insert represent cleaved PARP products from cells without paclitaxel treatment.

B: Percentage of viable cells after exposure to paclitaxel in MCF-7 cells, MCF-7/E1A-expressing cells (MCF-7-E1A), and a revertant of E1A-expressing clone (E1A-R) detected using MTT assay. The insert shows ΔPARP, and lanes 1, 5, and 9 in the insert represent cleaved PARP products from cells without paclitaxel treatment.

C: FACS analysis of sub-G1–phase apoptotic cells with or without exposure to 0.01μM paclitaxel for 24 hours. The symbols used are the same as those in panels A and B. DMSO, dimethyl sulfoxide.

Figure 2. E1A enhanced paclitaxel anti-tumor effect in nude mice *in vivo*.

A: Tumor volume of animals inoculated with MDA-MB-231-Vect (231-Vect) or MDA-MB-231-E1A (231-E1A) stable cells with or without treatment with paclitaxel. At least 5 animals were included in each group.

B: Immunohistochemical analysis of E1A expression in 231-Vect (a, b) and 231-E1A (c, d) cells growing in tissue culture *in vitro* (a and c, respectively) and in tumor tissue sample *in vivo* (b and d, respectively) after cells were inoculated into animal.

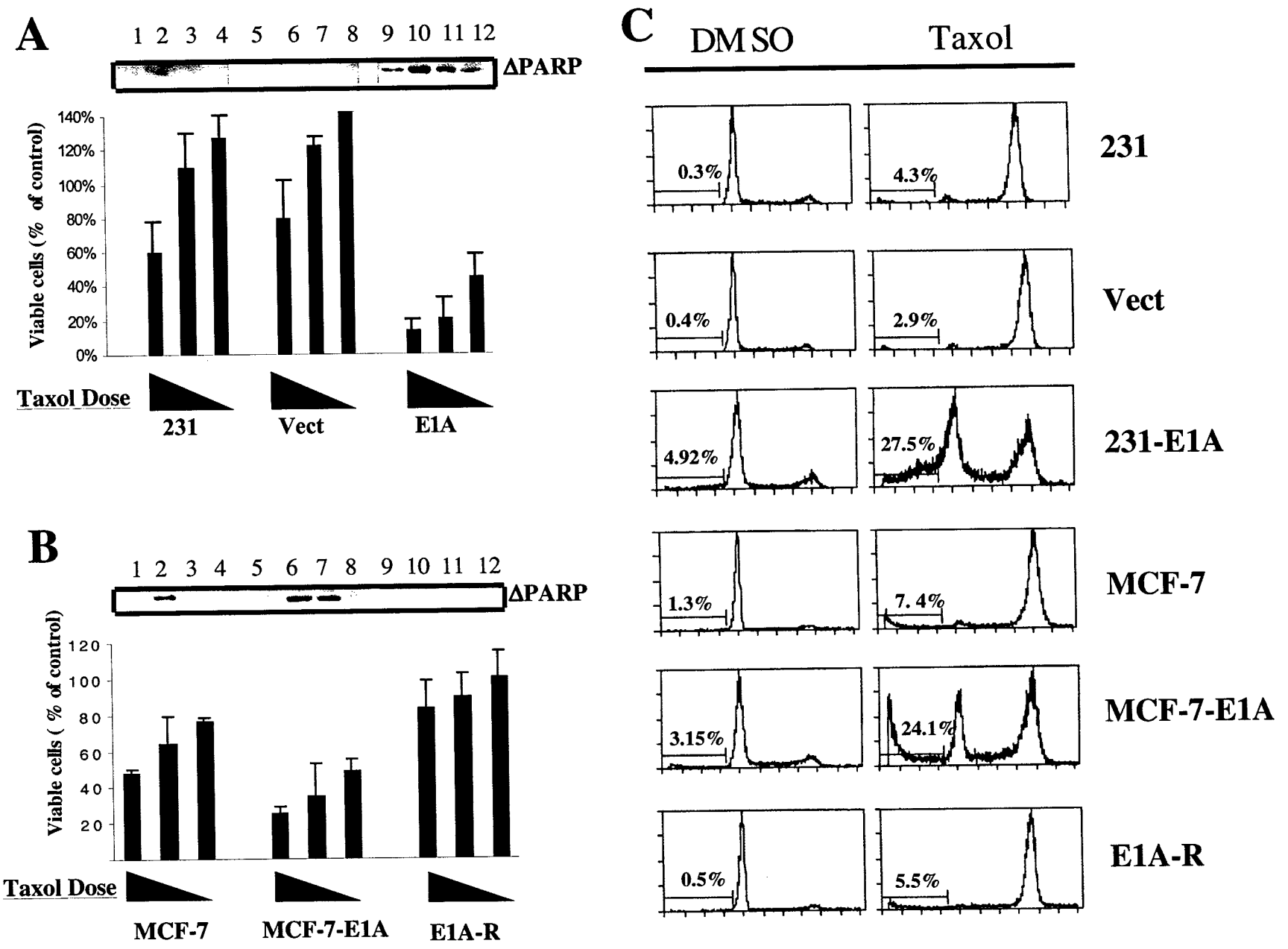
Figure 3. TUNEL assay of apoptotic cells in tumor tissue *in vivo*.

A: TUNEL labeling of apoptotic cells in tumor tissue samples obtained from 231-Vect or 231-E1A inoculated animals with (b and d) or without (a and c) treatment with paclitaxel.

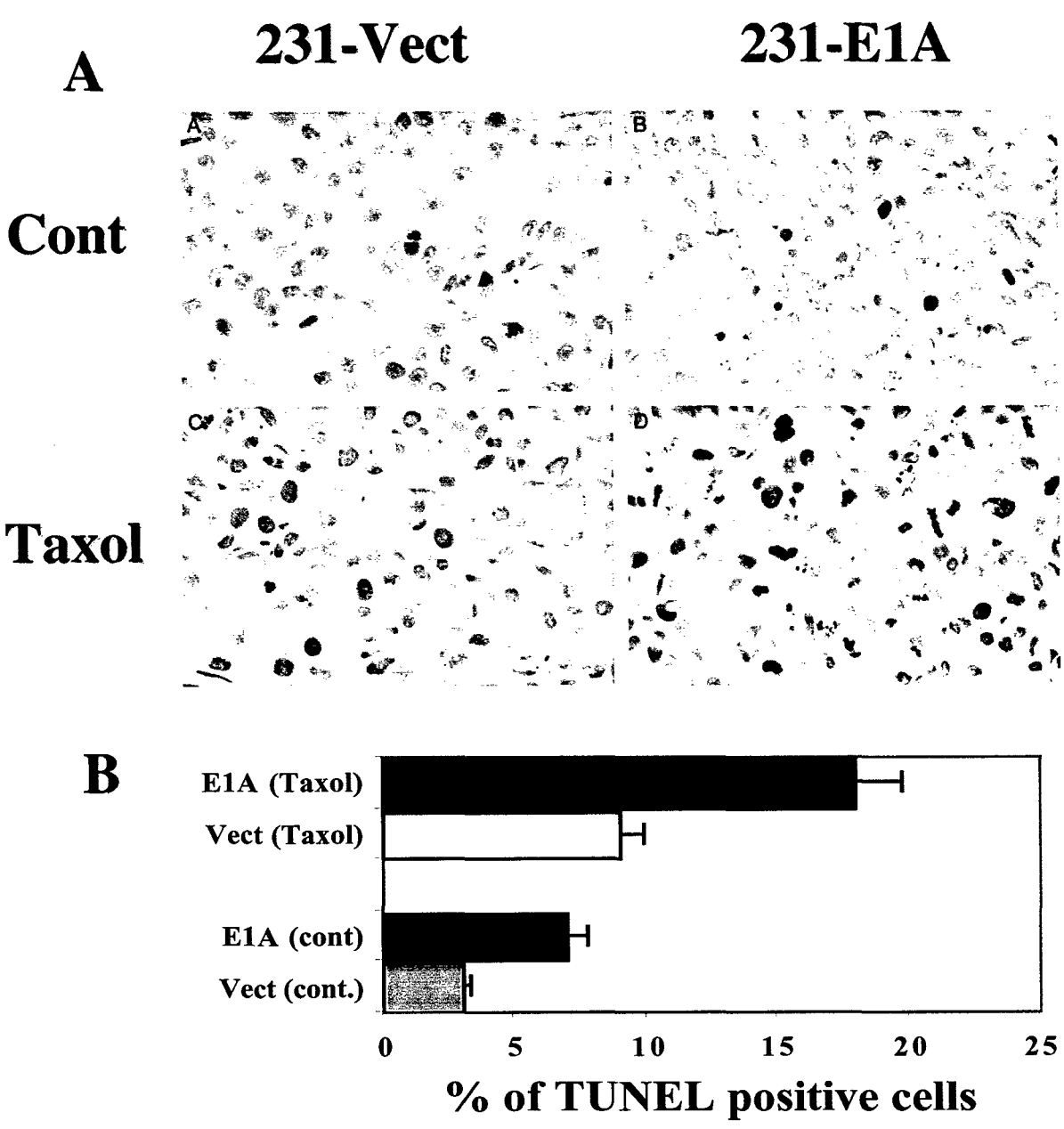
B: The percentage of TUNEL-positive cells in the tumor tissues samples obtained from 231-Vect and 231-E1A inoculated animals (each n=3) with or without treatment of paclitaxel.

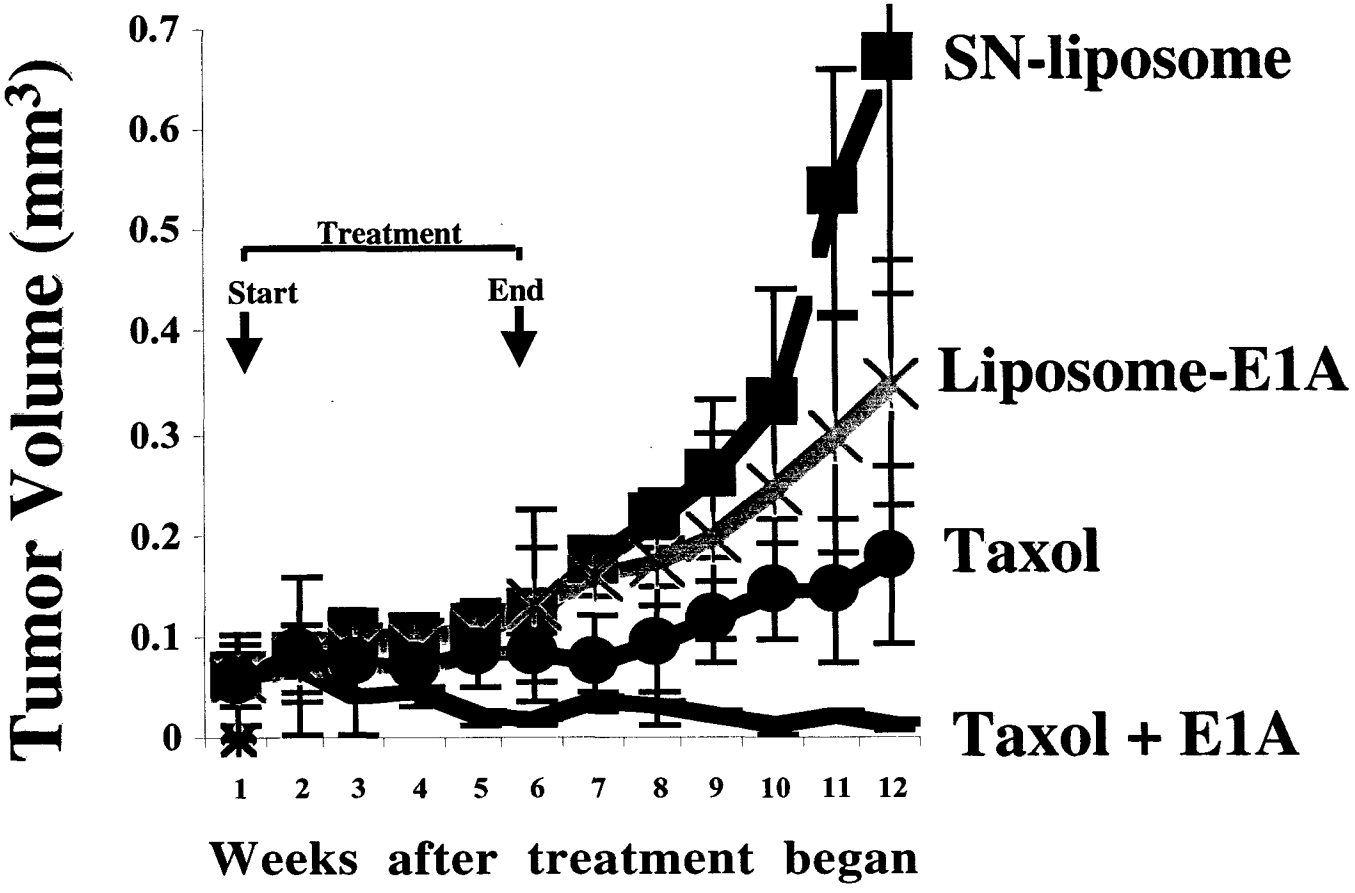
Figure 4. Tumor volume of animals during or after treatment with each of the regimes. Treatment groups included SN-liposome vehicle (■), SN-liposome-E1A (✱), paclitaxel alone (●), or paclitaxel plus SN-E1A (+). At least 7 animals were included in each group.

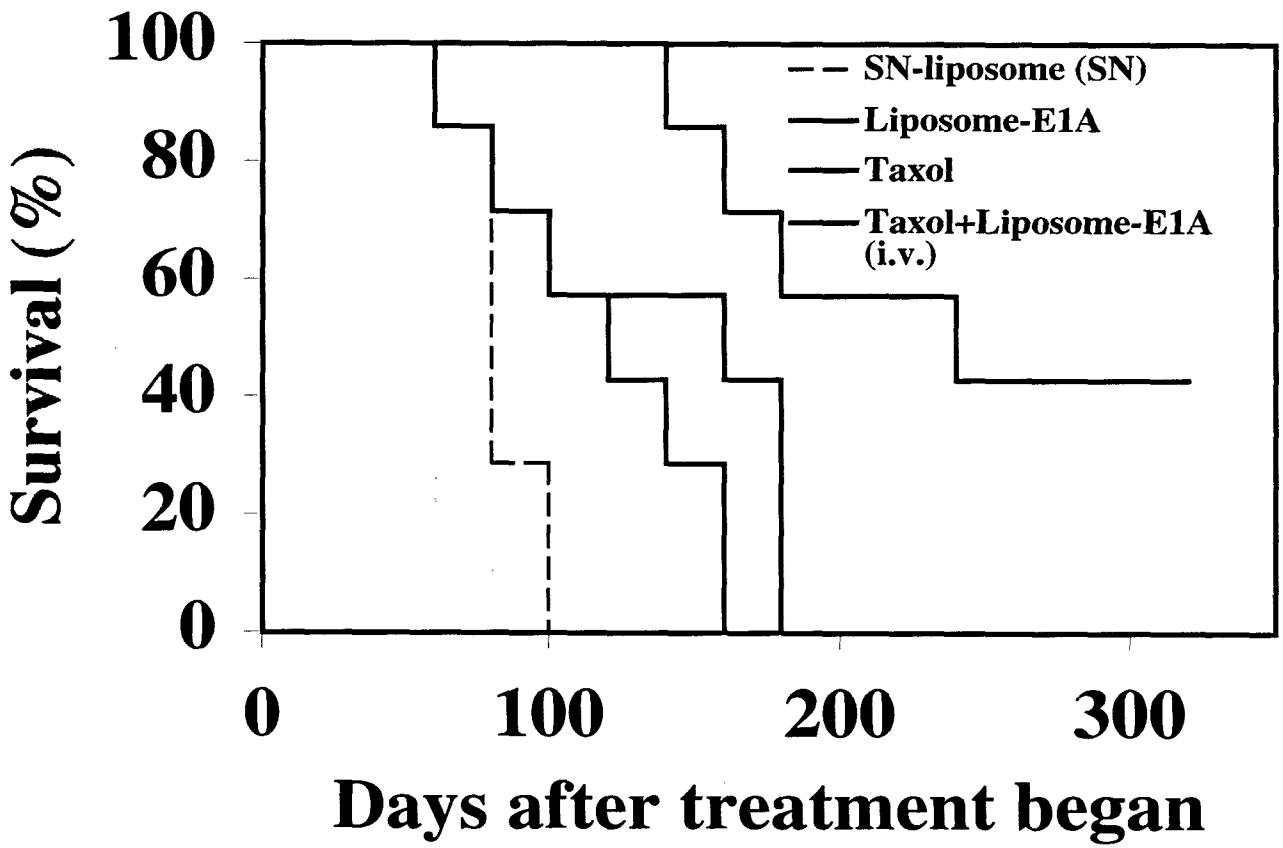
Figure 5. Survival curve of animals after treatment with systemic delivery of SN liposome alone, i.v. liposome-E1A alone, i.p. injection of paclitaxel, or combination of systemic liposome E1A and i.p. injection of paclitaxel.


A
1 2 3 4 5 6 7 8 9 10 11 12
ΔPARP
Viable cells (% of control)
140%
120%
100%
80%
60%
40%
20%
0%
Taxol Dose
231
Vect
E1A
B
1 2 3 4 5 6 7 8 9 10 11 12
ΔPARP
Viable cells (% of control)
120
100
80
60
40
20
Taxol Dose
MCF-7
MCF-7-E1A
E1A-R
C
DMSO
Taxol
0.3%
4.3%
231
0.4%
2.9%
Vect
4.92%
27.5%
231-E1A
1.3%
7. 4%
MCF-7
3.15%
24.1%
MCF-7-E1A
0.5%
5.5%
E1A-R

A
Tumor Volume (mm3)
0
0.2
0.4
0.6
0.8
231-Vect (cont.)
231-Vect (Taxol)
231-E1A (cont.)
231-E1A (Taxol)
B
Cell line (in vitro)
Tumor tissue
231-Vect
231-E1A
A
B
C
D


A
231-Vect
231-E1A
Cont
Taxol
A
B
C
D
B
E1A (Taxol)
Vect (Taxol)
E1A (cont)
Vect (cont.)
0
5
10
15
20
25
% of TUNEL positive cells


Tumor Volume (mm3)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Treatment
Start
End
1 2 3 4 5 6 7 8 9 10 11 12
Weeks after treatment began
SN-liposome
Liposome-E1A
Taxol
Taxol + E1A


100
80
60
40
20
0
Survival (%)
SN-liposome (SN)
Liposome-E1A
Taxol
Taxol+Liposome-E1A
(i.v.)
0
100
200
300
Days after treatment began

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AMERICAN SOCIETY of GENE THERAPY

March 21, 2003

Dr. Yong Liao
Mol. & Cellular Oncology
U.T. M.D. Anderson Cancer Center
1515 Holcombe Blvd., Box 079
Houston, TX 77030

Dear Dr. Liao:

On behalf of David Bodine, Chair of the ASGT Abstract Review Committee, it is my pleasure to inform you that your abstract, entitled "Enhanced Paclitaxel Cytotoxicity and Prolonged Animal Survival Rate by Non-Viral Mediated Systemic Delivery of E1A Gene in Orthotopic Xonograft Human Breast Cancer1," has been accepted for poster presentation during the 6th Annual Meeting of the American Society of Gene Therapy, June 4-8, 2003, at the Marriott Wardman Park Hotel in Washington, DC.

The details of your presentation are as follows:

Date:	Saturday, June 7, 2003
Session title:	Cancer Apoptosis
Session time:	4:00 PM to 7:00 PM
Location:	Exhibit Hall B & C
Final abstract number:	1074

Poster setup is from 10:00 am – 12:00 Noon on Thursday, June 5, and 7:00 am – 12:00 Noon on Friday, June 6, and Saturday, June 7, 2003; the posters will be available for viewing starting at 12:00 pm but authors do not need to be present until 4:00 pm. Please enter through Hall C. You must setup your poster during this time. The Exhibit Hall opens at 3:00 pm and those presenting posters on Thursday, June 5, should be present from 4:00 pm – 7:30 pm. For those presenting posters on Friday, June 6, and Saturday, June 7, 2003 you should be present from 4:00 pm to 7:00 pm. You must be present at your poster for the duration of the session in order to discuss your abstract with interested attendees. You must remove your poster at the close of the poster session that day. Posters remaining after 8:00 pm will be discarded.

Poster boards will be placed according to number. Your poster will be grouped with other abstracts covering similar topics. Abstract numbers will be affixed to the upper left-hand corner of the poster boards prior to the set-up time. You should prepare your poster to mount on a bulletin board surface that measures 7'6" wide by 3'8" high (2.3 meters wide by 1.1 meters high). Remember that the abstract number (11" w x 8 ½ h) will occupy the upper left-hand corner. Thumb tacks will be provided for your use. Include in your display a short and comprehensive review of the basic items of your study. This can be done by dividing your text into several clear sections including: title and authors, introduction, case reports/material/methods, figures/graphs/photos, results, conclusions, and major references. A good way to create your poster text is to use a laser printer and enlarge the text photographically until it can be easily read at a distance of about five feet.

All accepted abstracts will be published on the ASGT website beginning May 1, 2003. Abstracts will also be published in a supplement to the ASGT journal, *Molecular Therapy*, and will be distributed at the meeting.

Please Note: Abstract presenters must register for the meeting and pay the required registration fee. In order to make hotel reservations, please visit the ASGT website at http://www.asgt.org.

Please contact me if you have any questions. We look forward to a very successful meeting in Washington, DC, and appreciate your outstanding contribution to the scientific program.

Sincerely,

David Wood

David Wood
Director of Programs

Please select Print from the file menu to print your Abstract.

American Society of Gene Therapy

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Category: C7. Cancer - Apoptosis
Presentation Preferred: Poster
Prior Publication: No
Award: No, I am not applying for a trainee travel award.
Research funded by: Grant monies, This work was supported in part by NIH Grant RO1-CA58880 and the SPORE grant for ovarian cancer research from the National Institutes of Health (to M.-C. H.) and DAMD17-01-1-0300 from the United States Department of Defense Army Breast Cancer Research Program (to Y. L.).;
Conflict of Interest: No
Keyword 1: Cancer Gene Therapy **Keyword 2:** Non-Viral Gene Transfer **Keyword 3:** Apoptosis

Title: Enhanced paclitaxel cytotoxicity and prolonged animal survival rate by non-viral mediated systemic delivery of E1A gene in orthotopic xonograft human breast cancer1.

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Taxol is a promising frontline chemotherapeutic agent for the treatment of breast and ovarian cancers. To test whether E1A could sensitize low Her-2/neu-expressing MDA-MB-231 (p53 mutated) and MCF-7 (p53 wild-type) cell lines to Taxol-induced killing, we transfected wild type E1A into these cells and then treated with paclitaxel (Taxol) in vitro and in vivo. In the in vitro study, the E1A-expressing MDA-MB-231 (231-E1A) and MCF-7 (MCF-7-E1A) cells were exposed to different doses of Taxol and the cellular cytotoxicities of Taxol were evaluated by MTT cytotoxicity assays, PARP cleavage, and FACS analysis. The results showed that expression of E1A enhanced in vitro Taxol cytotoxicity, as compared to the control cells. For the in vivo study, we first compared the therapeutic efficacy of Taxol between orthotopic tumor models established with parental MDA-MB-231 versus 231-E1A stable cells, using tumor weight and apoptotic rate (TUNEL assay) as the parameters. We found Taxol was more effective in shrinking tumors and inducing apoptosis in tumor models established with stable 231-E1A cells than the control cells. We then tested whether E1A could directly enhance Taxol-induced killing in nude mice, by designing a systemic E1A gene therapy experiment using intravenous (i.v.) injection of the E1A gene via the mouse tail vein. We compared the therapeutic effects of E1A gene therapy with or without Taxol chemotherapy in the established orthotopic tumor model of animals inoculated with MDA-MB-231 cells, and found that a

combination of systemic E1A gene therapy and Taxol chemotherapy significantly enhanced the therapeutic efficacy and dramatically repressed tumor growth ($P < 0.01$). In addition, survival rates were significantly higher in animals treated with combination therapy than in the therapeutic control groups. Three of the seven animals treated with combination therapy achieved more than one year of tumor-free survival. Our data showed that expression of E1A significantly enhanced both chemosensitivity and an anti-tumor effect induced by Taxol as well as prolonging animal survival rates in the orthopic in vivo model.

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